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REMOVAL OF URANIUM AND MOLYBDENUM FROM URANIUM MINE WASTEWATERS BY ALGAE

by

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ABSTRACT

Uranium mine wastewaters at the Kerr McGee Corporation site near Grants, New Mexico, are treated in part by retention in a series of ponds which support a mixed bacterial and algal flora. The pond algae, predominately Chara, Spirogyra, and Oscillatoria, accumulate uranium and molybdenum from minewater. In Chara, average levels were 440-580 ppm uranium and 0-22 ppm molybdenum. Levels in filamentous algae varied over the year from 1090-2430 ppm uranium and 29-260 ppm molybdenum; the lower values were observed in summer and the higher in winter. In laboratory studies, Spirogyra removed more uranium and molybdenum from solution than Chara in 24-hour uptake. Grinding Spirogyra cells increased removal of uranium and molybdenum 2- to 3-fold over whole cells, with up to 82% removal of uranium and 37% removal of molybdenum. In long-term studies involving decaying algae in contact with sediment, the addition of algae to sediment promoted reducing conditions and usually increased sediment retention of uranium and molybdenum, but removal was reversible unless the model system contained high levels of organic material and sediment.

In the present algae pond system, the mass of algae is insufficient to remove significant levels of uranium and molybdenum from the high volume of minewater.

Increasing pond productivity would not produce enough algae to reduce the present levels of uranium and molybdenum by algal adsorption alone. However, greater productivity might enhance removal by increasing the amount of organic substrate available for sulfate-reducing bacteria, thus promoting hydrogen sulfide production in pond sediments, which would enhance trace contaminant removal.

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I. INTRODUCTION

A. Bioaccumulation of Heavy Metals

Wastewaters from industrial and mining operations often contain heavy metal pollutants in quantities too small to warrant recovery by conventional extractive techniques, but which must be removed to maintain water quality standards. Uranium minewaters, for example, may contain dilute concentrations of uranium, molybdenum, selenium, radium, and other associated ions (Brierley and Brierley, 1980). Lead mines discharge waters with low levels of lead, zinc, copper, cadmium and manganese (Gale and Wixson, 1979), and the zinc industry discharges zinc, cadmium, copper, and mercury in its effluents (Jackson, 1978). The possibility of using algae for water treatment has been considered as a low-cost alternative for removing such mixtures of metals, as the ability of algae to accumulate a range of heavy metals has been recognized (Jennett, Hassett, and Smith, 1979).

The bioaccumulation of heavy metals by microorganisms has been investigated extensively in relation to environmental pollution (Gadd and Griffiths, 1978; Leland et al., 1978). Analyses of algae collected in contaminated waters show concentrations of metals 10^2 to 10^4 times higher than in surrounding waters (McDuffy et al., 1976; Ray and White, 1976; Trollope and Evans, 1976). Good correlations between concentrations of copper and lead in seawater and benthic

algae were found by Seeliger and Edwards (1977), who suggested that algae might be used as indicators of environmental pollution. However, changes in the density and structure of naturally fluctuating populations, as well as physical and chemical differences in algal species, often obscure the relationship between concentrations in solution and concentrations in algae (Briand, Trucco, and Ramamoorthy, 1978; Knauer and Martin, 1973). Canterford, Buchanan and Ducker (1978) reported that concentrations of zinc, cadmium and lead in a marine diatom increased with higher external concentrations, but that the percentage of metals removed from water decreased at the higher concentrations. Coleman, Coleman and Rice (1971), using concentrations of up to 35 ppm zinc and 3 ppm cobalt, reported increased percentage uptake by freshwater algae with increasing concentrations of metals.

The study of microbial bioaccumulation in respect to treatment of polluted waters has been given increasing attention (Kelly, Norris and Brierley, 1979). Several water treatment systems have been developed to take advantage of the metal concentrating ability of algae. Gale and Wixson (1979) have investigated an artificial stream meander system which has been successful in removing lead, zinc and copper from lead mine effluents. Evidence indicated that the mixed algal flora, including Cladophora, Rhizoclonium, Hydrodictyon, and Spirogyra,

entrapped suspended mineral particles and accumulated metals by a cation exchange process. Investigation of zinc mine and smelter wastewaters by Jackson (1978) showed that planktonic algae blooms, stimulated by sewage effluents, removed zinc and some cadmium and copper from solution. A system of sequential ponds was suggested to maximize contact with the algae and to encourage sedimentation of dead algae so that accumulated metals could be sequestered by bacterially produced sulfides in the sediments. Filip et al. (1979) utilized a combined sand filtration and algae bioaccumulation system to remove copper, cadmium and chromium in wastewater primary treatment lagoons. Populations of Chlorella, Scenedesmus and Oscillatoria removed 70 to 90% of the 1.5 ppm cadmium and 2.3 ppm copper in solution, with 98 to 100% removal achieved in combination with the sand filter. A lower percentage uptake of 20% was observed for 15 ppm chromium in solution, and no increased removal was effected by the sand filter. Filip et al. (1979) postulated that binding sites for chromium had been saturated at the higher concentration.

B. Mechanisms of Uptake of Heavy Metals

Two types of mechanisms for uptake of heavy metals have been recognized in microorganisms: intracellular transport and binding of metals to cell surfaces. Intracellular uptake of heavy metals by energy-dependent

transport is metabolically controlled and utilizes pathways which exist for cellular regulation of trace metals and other essential ions, such as the K^+, Ca^{++}, and Mg^{++} transport systems, or the sulfate permease system which can transport oxyanions. The binding of metals to cell surfaces, on the other hand, involves the physical-chemical processes of adsorption and absorption. Kelly, Norris and Brierley (1979) concluded that retention of sorbed ions depends on formation of stable metal-organic complexes on cell surfaces, deposition of insoluble forms of the metals in cells, and incorporation of metals into cellular constituents.

Metabolically mediated uptake of several heavy metals has been demonstrated in algae. Zinc uptake in Chlorella fusca was characterized by initial rapid, reversible adsorption followed by long-term energy-dependent, largely irreversible uptake into the cell (Matzku and Broda, 1979). Similar patterns held for thallium and cadmium transport in Chlorella fusca (Solt, Paschinger and Broda, 1971; Mang and Trombella, 1978). Uptake of thallium may follow the pathway for potassium uptake, while cadmium seems to be taken up by the magnesium transport system. Manganese has also been shown to compete with cadmium uptake (Hart, Bertram and Scaife, 1979).

Nonmetabolic uptake of heavy metals plays an important

role in their bioaccumulation by algae. Algae have an overall negative charge (Bayne and Lawrence, 1972) which aids in binding cations to the cell surface. Veroy et al. (1980) found that lead and cadmium binding in the marine alga Eucheuma could be accounted for entirely by the formation of metal complexes with the negatively-charged cell wall polysaccharide carrageenan. In a study of localization of lead in field-exposed and laboratory test Cladophora, Gale and Wixson (1979) found that differences in lead accumulation on cell walls depended on length of exposure. Lead deposits were detectable on the surface of filaments when the cells were exposed to high concentrations of lead over a period of hours in laboratory tests. However, field-exposed specimens and laboratory specimens grown in lead solution over a period of weeks showed deposits only within the cell wall and capsular material, and none on the cell surface. The contrast between short- and long-term exposure suggests that an exchange absorption was involved in the incorporation of lead into cell wall constituents, while adsorption to the surface accounted for short-term uptake.

Extracellular material can also accumulate heavy metals. In the semicolonial marine alga Phaeocystis, whose individual cells are bound by a slime matrix, zinc accumulated in the colony matrix but was substantially released on disruption of the colonies, while manganese

was taken up and retained extracellularly (Morris, 1971). Hodge, Koide and Goldberg (1979) have suggested that the organic extracellular coating of marine macroalgae was involved in retention of particulate uranium, polonium and plutonium.

The phenomenon of increased adsorption of metals in dead over living cells has been observed in marine macroalgae for zinc but not cesium (Gutknecht, 1965), in marine phytoplankton for thallium but not potassium (Cushing and Watson, 1968), and in Chlorella regularis fpr UO_2^{++} (Horikoshi, Nakajima and Sakaguchi, 1979). Horikoshi et al. (1979) suggested that the increased uptake of UO_2^{++} in the heat-killed Chlorella regularis was due to an increase in adsorption sites on the disrupted cell walls rather than to an inhibition of cellular regulatory processes, since whole cells treated with metabolic inhibitors adsorbed UO_2^{++} at the same lower levels as the metabolizing whole cells. Adsorbed uranium was associated with cell wall and cell membrane fractions. Ferguson and Bubela (1974) used suspensions of fresh particulate material from Chlorella, Ulothrix and Chlamydomonas to accumulate copper, lead and zinc. At higher concentrations of metals, formation of metal-organic complexes produced deviations from monolayer adsorption behavior, so that higher percentages of metals were removed from solution. The ability of the particulate

suspensions to accumulate metals was considered good evidence for the ability of algae to influence the formation of metal-rich sediments.

C. Algae, Metal and Sediment Interactions

In pond and stream systems, the retention of heavy metals accumulated by either living or dead algal material depends on formation of insoluble metal-organic complexes and, perhaps more important, on the interactions between metals, algae and sediment.

Algae cells come in contact with sediments primarily as decomposing material. In the water treatment system studied by Jackson (1978), dying algae sank to the bottom, carrying accumulated metals into the sediment. Hydrogen sulfide produced by bacteria during decomposition of the algae could sequester the metals as insoluble metal sulfides. Zinc, copper, cadmium and mercury were associated more strongly in the sediments with sulfide than with organic carbon.

Studies on aerobic and anaerobic decomposition of a wide range of algae types indicate that both processes proceed at about the same rate and result in mineralization of around 60% of cell material, while the remaining fraction persists as particulate material with similar carbon content to the original cells (Fallon and Brock, 1979; Foree and McCarty, 1970; Seeliger and Edwards, 1979; Ulén, 1978). This refractory material could be a source of organic material for long-term complexing of metals in sediments.

Relatively few studies on algal bioaccumulation of heavy metals have considered the fate of the metals as the algae die and decay. Filip et al. (1979) observed release of 66% of accumulated cadmium and copper and 53% of chromium in decaying algae cultures after one week of exposure to the metals in growing cultures. Jennett, Smith and Hassett (1979) noted slow release of lead from dead or dying Chlamydomonas cells after a short accumulation period; mercury accumulated by Nostoc showed no release. Seeliger and Edwards (1979) followed the release of copper in decomposing red marine algae. During the decomposition period of 100 days, 30 to 60% of the copper accumulated previously be the growing algae was retained in refractory material and in particulate detritus derived from the decaying algae. The remaining fraction of accumulated copper was released primarily in dissolved organic material and to a lesser extent as inorganic material. While the inorganic copper was available for readsorption by algae, the organic copper complexes were not taken up by algae. Such dissolved metal-organic complexes may render the metal less susceptible to bioaccumulation (Sunda and Lewis, 1978) and, in some cases, may increase the solubility of the metal from a bound form (Bloomfield and Kelso, 1973; Jenne and Luoma, 1977).

In a study on metal, algae and sediment interactions, Laube, Ramamoorthy and Kushner (1979) observed that after uptake of cadmium and copper by Anabaena, cell lysis

released the metals as organic complexes of high molecular weight. Whether these organic complexes could be readsorbed onto the sediment was not investigated. It was noted, however, that Anabaena cells accumulated metals far more readily from water than from sediment, based both on final levels of the metals derived from each source and on percentage of available metal removed from each compartment.

D. Objectives of Present Study

The Kerr McGee Corporation in the Ambrosia Lake District near Grants, New Mexico, has developed a treatment system for uranium mine wastewaters. Water from two underground mines is pumped through a series of settling and algae ponds, which support a mixed bacterial and algal flora. Concentrations of uranium, selenium, and molybdenum in the water and sediments of the pond system have been established (Brierley and Brierley, 1980).

The studies described in this thesis have been undertaken to identify the major algal populations in the ponds and to determine the extent of uranium and molybdenum accumulation in the algae in situ . Also, laboratory experiments have been conducted to study the ability of two selected algae, Chara and Spirogyra spp., to accumulate uranium and molybdenum on short- and long-term exposure, with emphasis on the long-term retention of the metals in the presence of pond sediment.

II. MATERIALS AND METHODS

A. Site Description

The wastewater treatment system consists of a series of ponds which receive water pumped from two underground mine sections at a rate of $2x10^6$ gallons a day per section (Figure 1). Barium chloride is added to water from both sections prior to entry into the algae ponds, in order to coprecipitate radium as radium sulfate. Section 35 water passes through an ion exchange plant to remove uranium prior to combining with section 36 water. The residence time for the combined waters in the three algae ponds is about 2.5 days, based on a volume of $9x10^6$ gallons ($1.25x10^5$ ft^3) in the three algae ponds divided by the daily input of $4x10^6$ gallons.

Table I gives average values for water and sediment parameters measured between 11/15/78 and 4/29/80 (Brierley and Brierley, unpublished data).

The pond sediments provide a reducing environment (Eh, -350 mV) for a large population of sulfate-reducing bacteria of the genera Desulfovibrio and Desulfotomaculum (Brierley and Brierley, 1980). In the summer months between June and September, the pond perimeters support populations of Typha, Juncus, and sedges, which die back in the winter months.

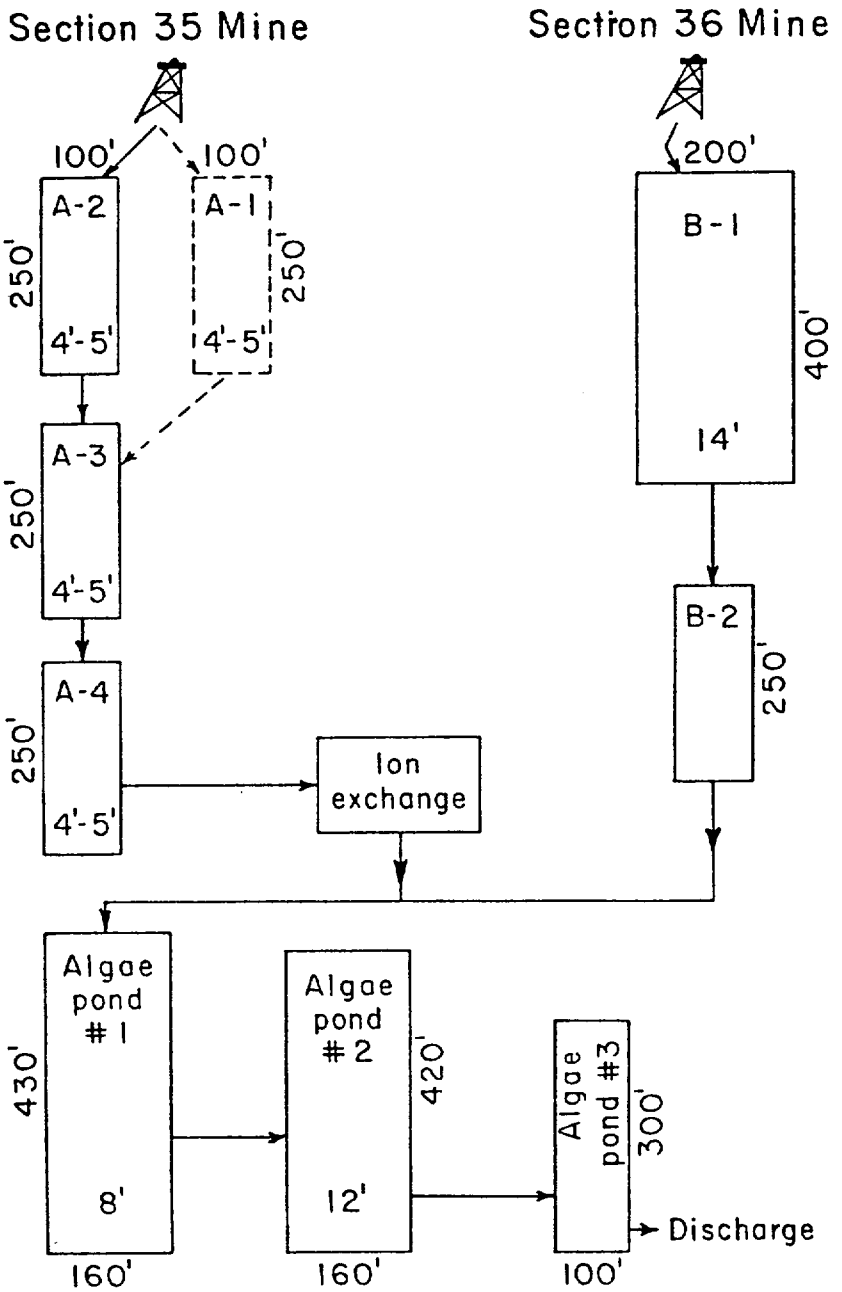

Figure 1. Schematic Representation of Pond System

TABLE I

CHEMICAL PARAMETERS IN POND WATER AND SEDIMENT

LOCATION	pH		CONDUCTIVITY (μmhos)		SUSPENDED SOLIDS (ppm)		NO_3 (ppm)	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
S-35 minewater	7.37	0.71	1280	225	129	113	1.10	0.50
A-1 sediment								
A-2 sediment								
A-2 effluent	7.56	0.63			39	23	1.10	0.46
A-3 sediment								
A-3 effluent	7.58	0.56			21	12	0.97	0.35
A-4 sediment								
A-4 effluent	7.60	0.48	1290	240	22	9	1.08	0.45
S-36 minewater	7.60	0.62	1320	220	266	243	0.60	0.64
B-1 sediment								
B-1 effluent*	7.19	0.30	1350	200	21	27	1.07	1.33
B-2 sediment								
B-2 effluent	7.76	0.51	1290	150	20	21	0.46	0.56
algae influent	7.67	0.47	1290	180	67	129	0.44	0.16
alg-1 sediment								
alg-1 effluent	7.66	0.30	1280	170	20	16	0.50	0.20
alg-2 sediment								
alg-2 effluent	7.68	0.46	1330	210	16	24	0.44	0.15
alg-3 sediment								
alg-3 effluent	7.81	0.45	1290	240	17	20	0.47	0.26
	n=6		n=5		n=7		n=6	

*Pond B-1 dry after 6/29/79

TABLE I--continued

CHEMICAL PARAMETERS IN POND WATER AND SEDIMENT

LOCATION	U(ppm)		Mo(ppm)		Se(ppm)		SO_4(ppm)**	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
S-35 minewater	4.6	1.2	2.0	0.5	0.026	0.014	720	50
A-1 sediment	918	200	202	226	0.105	0.084	3900	560
A-2 sediment	882	262	105	101	0.091	0.061	3000	190
A-2 effluent	5.1	0.5	1.9	0.5	0.017	0.005	720	50
A-3 sediment	1390	474	520	609	0.090	0.050	9100	2500
A-3 effluent	5.0	0.5	1.9	0.5	0.045	0.064	740	100
A-4 sediment	1634	857	420	679	0.063	0.037	4600	2600
A-4 effluent	4.4	0.8	1.9	0.4	0.022	0.007	690	50
S-36 minewater	3.4	3.4	0.04	0.05	0.014	0.023	680	60
B-1 sediment	3128	2595	25	64	0.041	0.053	3500	2500
B-1 effluent*	1.1	0.6	0.04	0.02	0.010	0.013	720	30
B-2 sediment	1488	1493	70	84	0.039	0.024	4100	400
B-2 effluent	1.0	0.7	0.05	0.02	0.005	0.004	680	60
algae influent	0.9	0.5	0.9	0.3	0.009	0.003	700	50
alg-1 sediment	1512	643	272	160	0.064	0.038	10800	2200
alg-1 effluent	1.0	0.7	1.0	0.3	0.011	0.003	700	50
alg-2 sediment	963	386	298	493	0.026	0.015	23700	12400
alg-2 effluent	0.8	0.4	0.9	0.3	0.010	0.004	700	50
alg-3 sediment	1097	283	371	131	0.073	0.042	12300	2500
alg-3 effluent	0.7	0.3	0.8	0.4	0.010	0.003	700	50
	n=6		eff n=8		n=7		eff n=7	
			sed n=6				sed n=4	

*Pond B-1 dry after 6/29/79

**Values represent SO_4(ppm) in water and total S (ppm) in sediment

B. Collection Procedure

Algae samples were collected from all three algae ponds on 9/25/79, 1/19/80, and 4/29/80. Samples were collected from the edge of the ponds, given a preliminary wash in pond water to remove excess particulate material, and stored in nonsterile polyethylene bottles on ice for transport to the laboratory. After further washing in deionized water, the samples were either dried for metal analyses or transferred to aquaria for laboratory culture. The most prominent algae were identified to genus using the keys of Prescott (1954) and Smith (1950).

C. Laboratory Culture

The algae were grown in mixed culture in 20 l aquaria. Shen's medium (Shen, 1971) was generally used for culture, with 0.2 g l^{-1} $NaHCO_3$ added to Chara cultures (Forsberg, 1965). About 1% activated charcoal was added to the media to remove possibly toxic growth byproducts (Shen, 1971). The algae were grown without aeration.

Table II gives nutrient concentrations for Shen's medium as well as for other media used in laboratory tests.

Illumination was provided continuously (Forsberg, 1965) by 2 22" GE "Gro and Sho" flourescent lights placed 12" from the water surface, giving 35-40 fc illumination. The temperature, which ranged from 18°C to 25°C, was usually 23°C.

D. Algae Digestions

In order to determine metal content in field-collected

TABLE II

MEDIA USED IN LABORATORY CULTURE OF ALGAE

1. Modified Bristol's Medium*

	concentration in medium	stock solution	amount stock solution used
$NaNO_3$	0.25 g l^{-1}	10 g/ 400 ml	10 ml l^{-1}
$CaCl_2 \cdot 2H_2O$	0.25	10 g/ 400 ml	10
$MgSO_4 \cdot 7H_2O$	0.075	3 g/ 400 ml	10
K_2HPO_4	0.075	3 g/ 400 ml	10
KH_2PO_4	0.018	0.7 g/ 400 ml	10
NaCl	0.025	1 g/ 400 ml	10
$FeSO_4 \cdot 7H_2O$	in 1 ml l^{-1} H_2SO_4	2.5 g/1000 ml	1

2. Bold Basal Medium*

a.	$NaNO_3$	0.25 g l^{-1}	10 g/ 400 ml	10 ml l^{-1}
	$CaCl_2 \cdot 2H_2O$	0.025	1 g/ 400 ml	10
	$MgSO_4 \cdot 7H_2O$	0.075	3 g/ 400 ml	10
	K_2HPO_4	0.075	3 g/ 400 ml	10
	KH_2PO_4	0.18	7 g/ 400 ml	10
	NaCl	0.025	1 g/ 400 ml	10
b.	EDTA	0.05	50 g/1000 ml	1
	KOH		31 g	
c.	$FeSO_4 \cdot 7H_2O$	0.005	4.98g/1000 ml	1
	H_2SO_4		1 ml	
d.	H_3BO_4	0.011	11.42g/1000 ml	1
e.	micronutrient solution			1
	$ZnSO_4 \cdot 7H_2O$		8.82g/1000 ml	
	$MnCl_2 \cdot 4H_2O$		1.44	
	$CuSO_4 \cdot 5H_2O$		1.57	
	$Co(NO_3)_2 \cdot 6H_2O$		0.49	

TABLE II--continued

MEDIA USED IN LABORATORY CULTURE OF ALGAE

1. Shen's Medium**

	concentration in medium
$CO(NH_2)_2$	0.04 g l^{-1}
$CaCl_2 \cdot 2H_2O$	0.10 g
$MgSO_4 \cdot 7H_2O$	0.10 g
Na_2CO_3	0.02 g
$NaSiO_3$	0.01 g
K_2HPO_4	0.6 mg
KCl	0.05 g
Tris	0.50 g

stock solutions b, c, d, and e (Bold Basal), 1 ml each l^{-1}

*Stein (1973)
**Shen (1971)

algae, samples were prepared for uranium and molybdenum analysis by digestion with nitric acid and hydrogen peroxide.

Samples of 0.1 to 0.75 g dry weight were added to 10 ml concentrated HNO_3. After overnight incubation at 85°C, the digestion was completed by addition of 5 ml 30% H_2O_2 with heating at 135°C for 5 minutes. Samples were cooled, filtered through 0.45 µm Millipore filter and brought to 250 ml volume with distilled water. Blanks without algae were run as controls for the digestion procedure.

A test for recovery of the metals by the digestion process was run on samples of 0.25, 0.5, and 0.75 g air-dried Chara (9/25/79 collection), half of which were spiked with uranium ($UO_2SO_4 \cdot 3H_2O$) and molybdenum ($Na_2MoO_4 \cdot 2H_2O$) to produce 0.2 ppm concentrations of each metal in the final dilution volume. The unspiked samples were compared to the spiked samples to evaluate recovery of metals added to the algae.

E. Uranium Toxicity Test

While uptake studies in the laboratory initially were conducted using uranium and molybdenum concentrations close to those in the minewaters, higher concentrations were desirable to facilitate quantitative analyses of the metals. A toxicity test for uranium was run to determine a range of concentrations which could be used without interfering with algal growth.

Two series of concentrations were prepared, one using pond water collected from the algae ponds and filtered through 0.45 µm Millipore filter, and one using Shen's medium without EDTA or Tris. Tris and EDTA were omitted because of possible chelation effects which might mask toxicity (Good. et al., 1966; Sunda and Lewis, 1978). Concentrations of uranium used were 0, 10, 20, 50, and 100 ppm. 5 ppm molybdenum were added to the media in all but the flasks containing 0 ppm U. Molybdenum and uranium spikes were added to batches of media and the pH adjusted to 7.5 with 0.1N NaOH before dispensing so that each series of flasks at a given concentration contained the same preparation.

100 mg wet weight of Chara, Spirogyra or Oscillatoria were added to 50 ml media in 250 ml Erlenmeyer flasks, which were incubated in a 12 hour light/dark regime for two weeks. Growth of algae was monitered by visual comparison of flask contents. The pH was checked after 1 week.

Flasks without algae but containing the series of uranium spikes were kept under similar conditions and analysed for uranium and molybdenum content at the end of the test.

F. 24 - Hour Uptake of Molybdenum and Uranium

Removal of molybdenum and uranium from solution by algae was tested in 24-hour uptake experiments. Table III

summarizes the specific conditions under which each experiment was conducted. In each test, the algae were washed in deionized water, dried on paper towelling, and weighed out immediately. The wet weight in Table III is the amount actually weighed out, while the dry weight was taken from a representative aliquot of the algae being tested, which was dried overnight at 85°C and reweighed. Particulate material was produced by grinding the algae one minute in a Sorvall Omni-Mixer, in a modification of a procedure suggested by Ferguson and Bubela (1974). Suspensions were used immediately instead of being frozen, and suspensions generally were not centrifuged before use. In experiment 1, one set of Chara particulate suspensions was centrifuged at 14,500 rcf and the pellet material alone used for uptake, and one set was left uncentrifuged.

To prepare test flasks, media were dispensed into the Erlenmeyer flasks and the pH adjusted with 1N NaOH or 1N HCl. After the algae were added, the flasks were spiked with stock uranium ($UO_2SO_4 \cdot 3H_2O$) and molybdenum ($Na_2MoO_4 \cdot 2H_2O$) solutions to produce the required concentrations, and the pH readjusted. Flasks without algae were used as controls. The experiments were carried out in stationary flasks incubated 24 hours in light.

The amounts of metals remaining in solution were determined after filtering the media through 0.45 µm Millipore filters to remove algal material. It was necessary to

TABLE III

CONDITIONS OF 24-HOUR EXPERIMENTS

EXPT.	PPM U	PPM MO	ALGAE	AMT. ALGAE	MEDIA	AMT. MEDIA	PH
1	5	3	Chara	2.0 g*	1:10 Bristol's	200 ml	7.7
				(0.4 g**)			
2	10	3	Spirogyra	2.0 g	1:10 Bristol's	200 ml	7.7
				(0.2 g)			
3	10	2.5	Chara	1.0 g	Shen's or	100 ml	7.8
				(0.2 g)	pond water	100 ml	7.8
4	10	5	Spirogyra	1.0 g	Shen's or	100 ml	8.0
				(0.1 g)	pond water	100 ml	8.0

*wet weight

**dry weight

centrifuge the particulate suspensions for 10 minutes at 4,340 rcf before filtering. In experiment 3, the algal material was recovered and analysed for metal content.

G. Long-Term Studies

Long-term interactions between water, algae and sediment were modeled in the laboratory using 2 l culture vessels containing 1 l medium spiked with uranium and molybdenum, and growing algae cultures. Table IV summarizes conditions in four long-term experiments. Algae were added in amounts measured by wet weight, with comparable aliquots dried for the dry weight determination. Growth was monitered visually. The first three experiments involved a period of growth in light for the algae exposed to the metal solutions. In experiments II and III, the light incubation period was followed by a long period in the dark, during which two of the algae containers and two of the control containers were given additions of pond sediment. In experiment IV, the amounts of sediment and algae were increased relative to the amount of solution, and the light incubation period was omitted since the algae were added as dry material.

The pH of all tests was monitered, and Eh measurements were taken during the latter period of experiment IV. Concentrations of uranium and molybdenum in the media were monitered by periodic removal of 100 ml solution, which were filtered and acidified before metal analysis. The 100 ml aliquot was replaced with an equal volume of medium

TABLE IV

CONDITIONS OF LONG-TERM EXPERIMENTS

EXPT.	PPM U	PPM MO	ALGAE	AMT. ALGAE	MEDIA	PH	DAYS IN LIGHT	DAYS IN DARK	AMT. SEDIMENT
I	5	2	Spirogyra	15 g*	Bristol's	6.5	14	0	0
II	10	3	Chara	10 g (2.4 g**)	1:4 Bristol's	7.7	12	142	150 g*
III	5	2	Chara	5 g (1.1 g)	Shen's	7.8	14	152	150 g
			Spirogyra	5 g (0.4 g)					
IV	20	5	Chara	20 g***	pond water	8.1	0	190	1000 g

*wet weight

**dry weight

***added as dry material

containing the initial metal concentrations.

H. Analytical Procedures

Uranium concentrations in digested algae or in water samples were determined colorimetrically by the TOPO extraction procedure (Johnson and Florence, 1971). Replicate analyses for a given sample varied from 0 to 33% with a mean variation of 9% (based on 12 samples).

Molybdenum was assayed colorimetrically by the thiocyanate procedure (Meglen and Glaze, 1973). The analysis varied from 1 to 35% with a mean variation of 20% (based on 5 samples). Appendix A gives a detailed description of both the uranium and the molybdenum analyses.

Water samples from uptake or toxicity studies were generally diluted 1:10 or 1:20 before analysis, while digested algae samples were run at 1:2 or at no dilution.

I. SEM Studies

Algae specimens were prepared for microscopy by dehydration in the following ethanol:amyl acetate series: ethanol in distilled water 30%, 50%, 70%, 95%; 100% ethanol twice; amyl acetate in absolute ethanol 30%, 50%, 70%, 95%; 100% amyl acetate twice. Samples were left in each solution 15 minutes (Hayes, 1973). The samples were transferred to liquid carbon dioxide in a Ladd Critical Point Dryer for critical point drying. The dried samples were mounted onto stubs using nitrocellulose glue and were sputter-coated with carbon in a Denton Vacuum (DV-502) high vacuum

evaporator. The specimens were examined with a Hitachi HHS-2R scanning electron microscope.

Micrographs were taken on Polaroid Type 55 positive/ negative 4x5 Land film.

III. RESULTS

A. Algae Populations

The predominant algae in the ponds were the macrophytic green alga Chara, the filamentous green alga Spirogyra, and the filamentous blue-green alga Oscillatoria. Rhizoclonium, another filamentous green alga, was found at the inflow of algae pond 1 during the summer. Unidentified diatoms and unicellular green algae were associated with the floating mats of filamentous algae and with Chara; these associated algae were most abundant in the summer months.

The composition of the algal flora varied over the year. In the winter months of November, January and March, growth of algae was sparse. The floating algae were concentrated at the inflow and outflow of each pond. Oscillatoria tended to dominate the filamentous algae in the winter. Collections from April, June and September showed a higher proportion of Spirogyra. During the summer months, growth of the filamentous algae extended in patches out into the deeper sections of the ponds, although only pond 3 showed extensive coverage even in summer. Chara formed a dense mat of fronds over the pond floors during the entire year, but growth evidently took place mainly in the summer months. Winter vegetation of Chara was weathered and more mineralized than summer vegetation, suggesting that the winter material was a survival of summer growth.

Figures 2, 3 and 4 are scanning electron micrographs of Chara, Spirogyra , and Oscillatoria, respectively. The epiphytic diatoms are especially evident on Chara. Also, the heavy mineral coating on Chara can be seen, in contrast to the smooth filaments of Oscillatoria and Spirogyra.

B. Laboratory Culture

Algae cultures were grown initially in modified Bristol's medium (Table II), chosen because of its general applicability to a wide range of green algae. Repeated failure of Chara and limited growth of Spirogyra led to trials with other media.

Additions of trace elements and EDTA as specified in Bold Basal medium (Table II) did not improve Chara or Spirogyra growth and seemed detrimental to Oscillatoria. A unialgal culture of Oscillatoria was maintained for a year in modified Bristol's medium but would die when transferred to Bold Basal medium.

The medium used successfully for Chara and Spirogyra culture was Shen's medium (Table II), developed specifically for Chara based on the alga's requirement for extremely low phosphorus concentration (Shen, 1971). Forsberg (1965) has reported that phosphorus concentrations above 25 $\mu g\ l^{-1}$ are inhibitory to Chara. Collections of Chara and Spirogyra transferred from field collections directly to aquaria containing Shen's medium could be maintained three to four weeks providing the initial

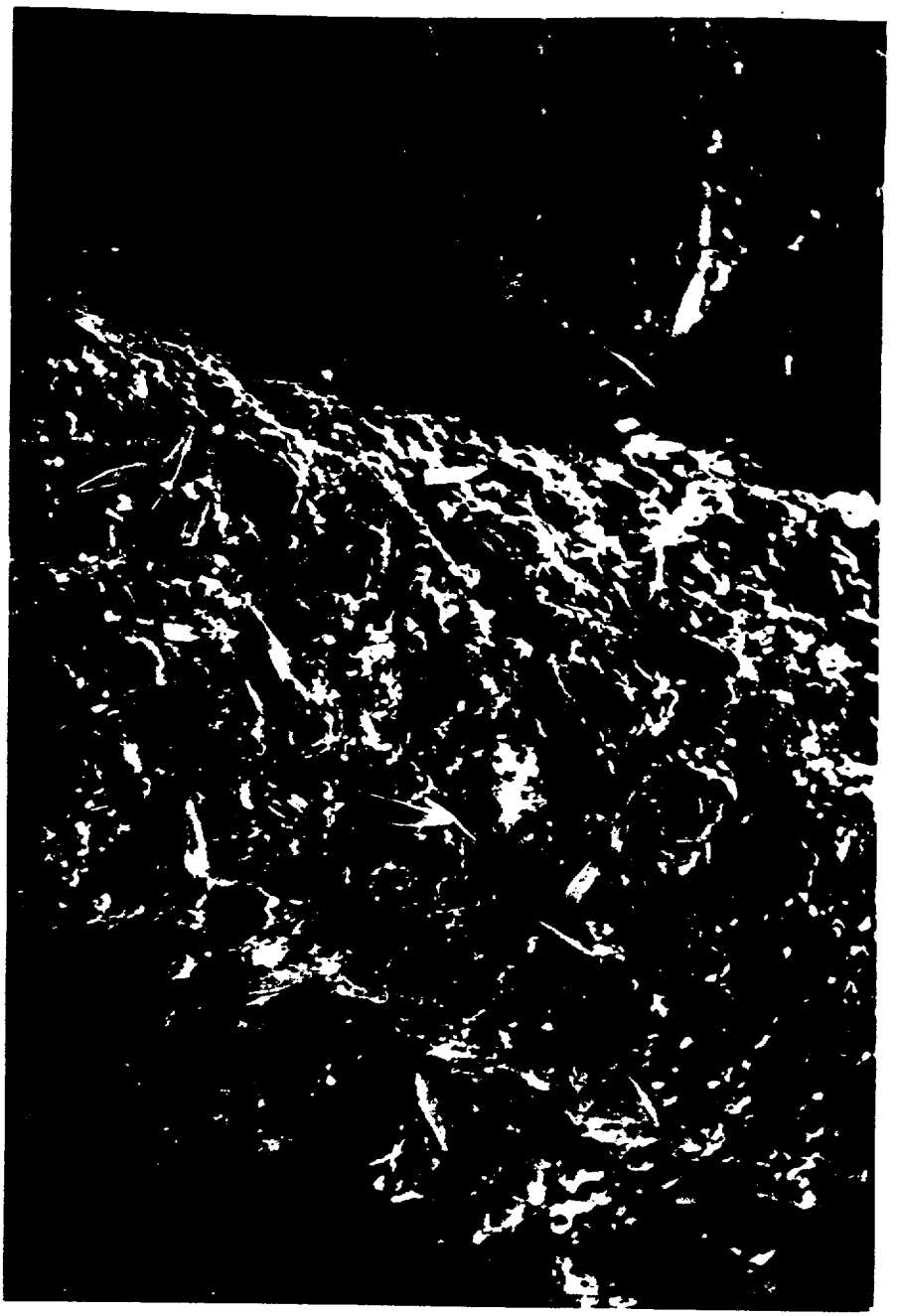

Figure 2. Chara, scanning electron micrograph (X350)

Figure 3. Spirogyra, scanning electron micrograph (X875)

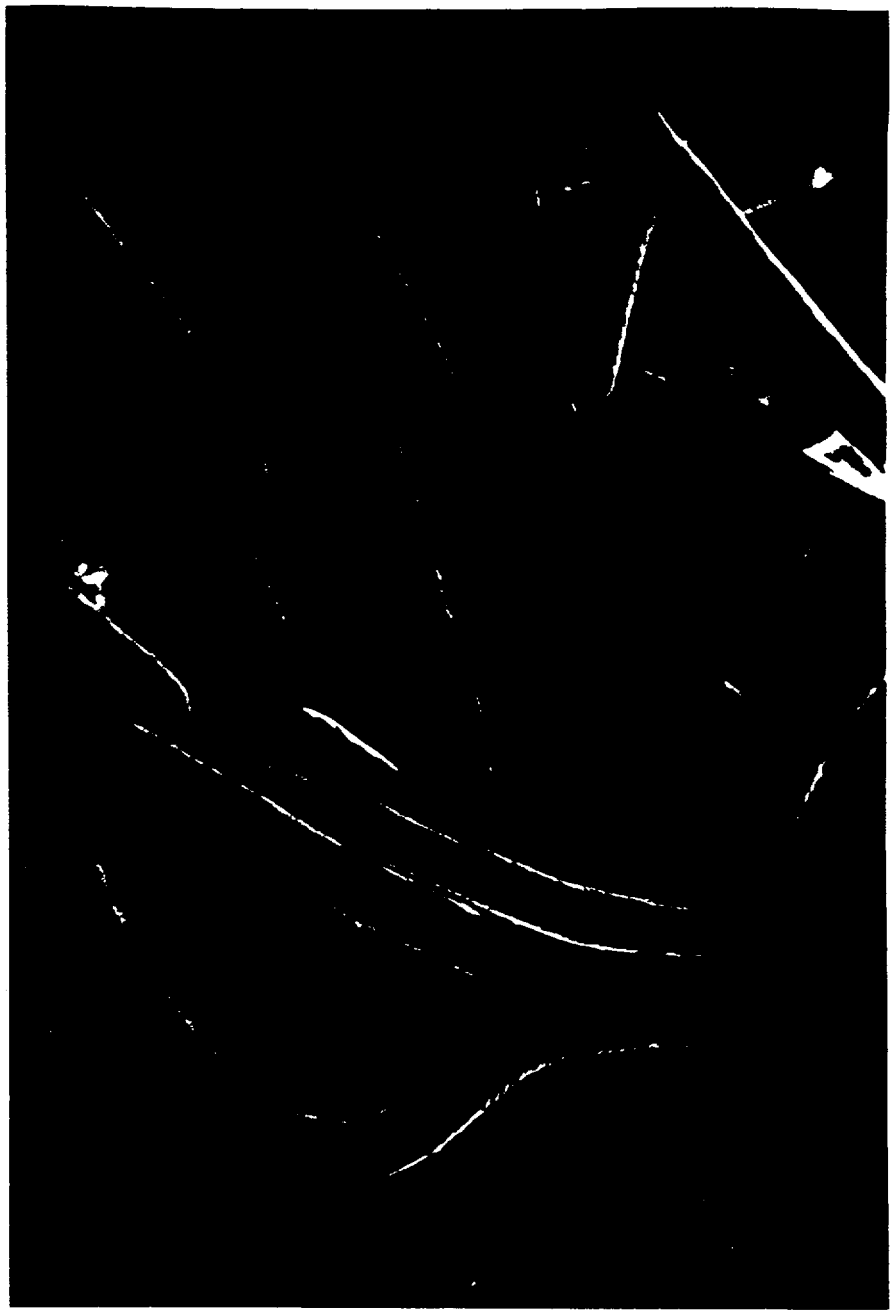

Figure 4. Oscillatoria, scanning electron micrograph (X1575)

aliquot was sparse. Subsequent overgrowth of other algae in the aquaria replaced Spirogyra and Chara. A typical succession of growth in an aquarium initially stocked with Chara consisted of Oscillatoria on the aquarium walls and a surface network of blue-green Lyngbya filaments mixed with unicellular and colonial green algae. Festoons of filamentous Ulothrix appeared after several weeks. Although Chara had grown well in the aquarium initially, after growth of the successional algae only a few etiolated shoots were formed. The cultures of Chara and Spirogyra used for laboratory tests were removed from the aquaria before this stage of overgrowth had begun.

C. Metal Levels in Field-Collected Algae

Table V gives results for the test for recovery of metals in the digestion process which was described in section II D. Molybdenum was less likely to be recovered in the analysis than uranium. The inability to recover the spike amount of molybdenum was evident in solutions both with and without algal material and probably was due to loss by precipitation or adsorption onto flask walls rather than to incomplete digestion of the algae. Recovery of uranium was fairly consistent over the range of sample sizes and was unaffected by the addition of the uranium spike.

The results of analyses for three collections of algae are summarized in Table VI. Table VII gives the means for

TABLE V

TEST FOR RECOVERY OF URANIUM AND MOLYBDENUM IN DIGESTION OF ALGAE

ALGAE SAMPLE DRY WEIGHT	PPM U SPIKE*	PPM U IN SAMPLE	PPM U IN ALGAE**	PPM MO SPIKE*	PPM MO IN SAMPLE	PPM MO IN ALGAE**
0 g	0.2	0.29	-	0.2	0.13	-
25 g	0.2	0.62±0.06	420	0.2	0.10±0.08	0
25 g	0	0.34±0.10	340	0	0	0
50 g	0.2	0.99	400	0.2	0.20	0
50 g	0	0.55±0.22	275	0	0.01±0.00	5
75 g	0.2	0.95±0.06	250	0.2	0.10±0.09	0
75 g	0	0.81±0.05	270	0	0.06±0.04	20

*Samples were spiked before digestion with uranium and molybdenum or were left unspiked.

**Concentrations in algae were calculated by the formula

$$\frac{(\text{ppm sample} - \text{ppm spike}) \times 0.25\ 1 \times 10^{3}}{\text{g algae}} = \text{ppm in algae}$$

TABLE VI

URANIUM AND MOLYBDENUM LEVELS IN FIELD-COLLECTED ALGAE

DATE OF COLLECTION:	9/25/79	1/24/80	4/29/80
A. URANIUM (ppm)			
Chara			
pond 2	544±439 (7)*	580 (1)	375±25 (2)
pond 3	330±285 (2)	NC**	580 (1)
Spirogyra, Oscillatoria			
pond 1	NC	1850±400 (4)	2015±15 (2)
pond 2	870±90 (3)	2670±530 (4)	1700±420 (3)
pond 3	NC	3100±300 (2)	1150 (1)
Rhizoclonium			
pond 1	1420±50 (2)	NC	1700±100 (2)
B. MOLYBDENUM (ppm)			
Chara			
pond 2	22±20 (2)	0 (2)	40±4 (2)
pond 3	0 (2)	NC	4±0 (2)
Spirogyra, Oscillatoria			
pond 1	NC	320±150 (4)	70±17 (2)
pond 2	27±16 (3)	270±200 (4)	53±8 (4)
pond 3	NC	120±50 (2)	35±1 (2)
Rhizoclonium			
pond 1	31±6 (2)	NC	30±11 (2)

*Number in parenthesis indicates sample size

**NC = no collection

TABLE VII. URANIUM AND MOLYBDENUM LEVELS IN FIELD-COLLECTED ALGAE AVERAGED OVER PONDS 1, 2, AND 3

DATE OF COLLECTION:	9/25/79		1/24/80		4/29/80	
	U PPM	MO PPM	U PPM	MO PPM	U PPM	MO PPM
Chara	500±390	16±19	580	0	440±120	22±21
Spirogyra Oscillatoria Rhizoclonium	1090±310	29±12	2430±670	260±190	1710±350	48±18

filamentous algae and for Chara calculated over all samples from each collection date, taking the three ponds as one collection. Levels of molybdenum and uranium were higher in filamentous algae than in Chara by 2 to 3 times for uranium and about 2 times for molybdenum. Wide variation in both molybdenum and uranium levels were found; uranium ranged from a low of 330 ppm (Chara, 9/25/79) to 3100 ppm (filamentous, 1/24/80), and molybdenum from undetectable levels in some Chara samples to a high of 320 ppm in the filamentous algae collected 1/24/80. Generally, in a given sample the uranium concentration was an order of magnitude higher than the molybdenum.

Some seasonal variation was seen in the filamentous algae but was not obvious in Chara. A one-way analysis of variance for uranium and molybdenum levels in filamentous algae by month showed significant variation at the .01 level (see Appendix B for degrees of freedom and F values, following the method of Snedecor and Cochran, 1967). The levels of uranium were significantly different for all months, with levels increasing in the order September('79)

April ('80), January ('80). Molybdenum concentrations were significantly higher in January ('80) over September ('79) and April ('80), but the difference between September and April was not significant. There was no correlation between the metal content of a sample and pond from which it was collected.

D. Uranium Toxicity

Table VIII summarizes the growth response of Spirogyra, Chara, and Oscillatoria to increasing concentrations of uranium. After 1 day of culture, Oscillatoria trichomes had spread out over the flask surface in pond 0 ppm and 10 ppm flasks, but not in Shen medium or in pond 50 ppm. No differences were seen among the Chara or Spirogyra flasks; even at 100 ppm, the cells were not lysed and looked viable. By day 3, some Spirogyra cultures were moribund (those marked with a minus sign in Table VIII), and Chara internodal cells had lysed in Shen medium. Oscillatoria cultures in pond water showed active growth even at 50 ppm.

By day 11, all cultures in Shen medium were dead or moribund. The medium had been modified by omission of EDTA and Tris, which evidently rendered the medium unfit for the algae, since even the cultures with no added uranium died. There were no significant differences in pH among the Shen medium flasks and the pond water, but trace elements in the medium might have been toxic in the absence of EDTA.

TABLE VIII

GROWTH OF ALGAE IN INCREASING CONCENTRATIONS OF URANIUM*

MEDIUM	PPM U	PPM MO	ALGAE	DAY 1	DAY 3	DAY 11	DAY 16	PH
Shen's	0	0	Chara	+	+	x	x	8.0
			Spirogyra	+	-	x	x	7.8
			Oscillatoria	-	-	x	x	7.9
Shen's	10	5	Chara	+	-	x	x	8.0
			Spirogyra	+	+	-	x	7.5
			Oscillatoria	-	-	x	x	7.5
Shen's	20	5	Chara	+	-	x	x	7.9
			Spirogyra	+	-	x	x	7.3
Shen's	50	5	Chara	+	+	x	x	8.0
			Spirogyra	+	-	x	x	7.3
			Oscillatoria	-	-	x	x	7.7
Shen's	100	5	Chara	+	-	x	x	8.0
			Spirogyra	+	-	x	x	7.3
pond water	0	0	Chara	+	+	+	+	8.2
			Spirogyra	+	+	+	+	8.6
			Oscillatoria	+	+	+	+	8.2
pond water	10	5	Chara	+	+	+	+	8.3
			Spirogyra	+	+	+	+	8.5
			Oscillatoria	+	+	+	+	8.3
pond water	20	5	Chara	+	+	+	+	8.3
			Spirogyra	+	+	+	+	8.9

TABLE VIII--continued

GROWTH OF ALGAE IN INCREASING CONCENTRATIONS OF URANIUM

MEDIUM	PPM U	PPM MO	ALGAE	DAY 1	DAY 3	DAY 11	DAY 16	PH
pond water	50	5	Chara	+	+	+	-	8.3
			Spirogyra	+	+	+	+	8.8
			Oscillatoria	-	+	+	+	8.5
pond water	100	5	Chara	+	+	-	x	8.2
			Spirogyra	+	+	+	+	8.7

*Symbols represent good growth (+), poor growth (-), or dead cultures (x).

After 15 days' culture, Chara was still healthy in appearance in pond water at 0 ppm, 10 ppm, and 20 ppm uranium. Cultures at 50 ppm were moribund and those at 100 ppm were dead. Both Oscillatoria and Spirogyra maintained viable cells at the highest concentrations of uranium to which they were exposed. A microscopic examination of Spirogyra cells taken from the flasks revealed intact, well-formed cells containing fully-formed chloroplasts at 0 ppm, 10 ppm, and 20 ppm uranium. At 50 ppm and 100 ppm, chloroplasts in mid-strand, that is in cells which had grown before contact with the high uranium concentrations, were normal, but at the growing tips of the strands, chloroplasts showed evidence of disintegration. Growth at the tips was characterized by abnormal cell division, giving rise to mid-strand side branches uncharacteristic of Spirogyra, and by abnormal cell wall formation. Although cellular metabolism was not totally impaired at the higher concentrations, the abnormal cell growth suggested that cultures could not be maintained at concentrations much above 20 ppm uranium, and this is the concentration that was used in subsequent laboratory experiments.

E. 24- Hour Uptake

Uptake of uranium and molybdenum on a short-term basis was studied in Chara and Spirogyra in 24-hour laboratory uptake experiments. Results for the tests are given in Table IX. Only one experiment (3A, Table IX) showed

detectable uptake of either uranium or molybdenum in Chara. The detection of uranium and molybdenum in field-collected specimens indicates that Chara does have the ability to accumulate the metals from solution, but the conditions of the short-term uptake studies evidently did not allow uptake or only at levels below detection. The excess of uranium in the Chara preparations in Experiment 1 (Table IX) probably is not due to release of uranium from the algae cells. Analysis of samples of Chara before and after treatment gave 300 ppm uranium in the untreated algae, 930 ppm in the whole, 1950 ppm in centrifuged particulate, and 1190 ppm in particulate preparations after incubation. Since the treated algae had higher levels of uranium than before treatment, it is more likely that the value for the control was low due to precipitation of uranium rather than that the algae had released uranium. Increasing the cell surface by disintigration of Chara cells had no effect on removal of metals from solution.

Spirogyra preparations adsorbed both uranium and molybdenum from solution (Table IX). In every case, disruption of the cells by omni-mixing increased the adsorption. The pattern of removal of both metals is strikingly similar in the three different solutions employed in the tests. Disrupting the cells increased uptake of uranium 2- to 3-fold in each case. Increasing the external concentration

TABLE IX

24-HOUR UPTAKE OF MO AND U BY CHARA AND SPIROGYRA

EXPERIMENT	PPM U	% REMOVED	MG U/G ALGAE*	PPM MO	% REMOVED	MG MO/G ALGAE*
1 Chara in Bristol's						
Control	2.5±0.4			2.9±0.0		
Whole	18.4±2.0	0		3.0±0.0	0	
Centrifuged Particulate	18.0±1.3	0		2.8±0.1	0	
Particulate	20.8±0.4	0		2.8±0.3	0	
3A Chara in Shen's						
Control	10.1±0.1			2.2±0.1		
Whole	8.2±2.6	19%	0.9 mg/g	2.7±0.4	0	
Particulate	8.3±2.5	18%	0.9 mg/g	2.2±0.2	0	
3B Chara in pond water						
Control	9.7±0.4			2.4±0.1		
Whole	9.9±1.3	0		2.5±0.1	0	
Particulate	10.0±1.1	0		2.5±0.2	0	

TABLE IX--continued

24-HOUR UPTAKE OF MO AND U BY CHARA AND SPIROGYRA

EXPERIMENT	PPM U	% REMOVED	MG U/G ALGAE*	PPM MO	% REMOVED	MG MO/G ALGAE*
2 Spirogyra in Bristol's						
Control	10.9±1.4			2.5±0.1		
Whole	7.8±0.0	28%	3.1 mg/g	2.7±0.0	13%	0.4 mg/g
Particulate	1.9±0.5	82%	9.0 mg/g	2.4±0.3	22%	0.7 mg/g
4A Spirogyra in Shen's						
Control	21.4±0.8			4.9±0.1		
Whole	13.5±0.1	37%	7.9 mg/g	4.8±1.2	0	
Particulate	6.2±0.1	71%	15.2 mg/g	4.2±0.6	14%	0.7 mg/g
4B Spirogyra in pond water						
Control	23.9±1.7			4.5±0.1		
Whole	17.9±0.3	25%	6.0 mg/g	4.9±0.2	0	
Particulate	5.2±1.2	78%	18.7 mg/g	4.3 0.5	4%	0.2 mg/g

*Obtained by dividing the amount in mg of U or Mo removed from solution by the dry weight of algae per flask

of uranium did not decrease the percentage uptake, so that the total amount of adsorbed uranium was higher at the higher external concentrations. In the case of molybdenum, doubling the concentration in solution did not increase the amount adsorbed, and in fact the percentage uptake was lower at the higher external concentrations. Disruption of cells increased the amount adsorbed, though on a smaller scale than for uranium.

The material used for the 24-hour studies consisted of living cells, which were left intact in the whole preparations and killed only in the omni-mixed preparations. After the 24-hour incubation period, the whole preparations retained their cellular integrity and appeared normal. The particulate suspensions began to lose their chlorophyll pigment after 24 hours. While the solutions from whole preparations were colorless after filtering through 0.45 μm Millipore filter, the solutions from the disintegrated cells often were tinted pale brown, which may be an indication of release of organic acids (Jackson, Jonasson and Skippen, 1978).

F. Long-Term Uptake

The effects of cell growth and subsequent decay on metal uptake by algae were followed in a series of long-term studies, most of which also involved the interaction between algal material and pond sediment during decay. Tables X-XIII give the results from the four long-term

experiments.

Experiment I (Table X) involved Spirogyra without added sediment or prolonged decay period. The cultures grew well for 5 days, after which the algae decayed into fine brown-colored particles. The Bristol's medium used in this test seemed to have precipitated most of the 5 ppm uranium spike, since the day 0 sample taken directly after addition of the uranium contained only 1.2 ppm in the control without algae. Molybdenum, however, remained in solution. No decrease in either uranium or molybdenum concentrations in the media containing the algae was observed until day 5, which coincided with the onset of decay. After 14 days, the levels of uranium and molybdenum in the containers with algae were reduced about 40% from the day 0 levels.

Experiment II (Table XI) was designed to study the possibility of increased uptake in decaying algae as suggested by the results from the first experiment. Cultures of Chara were allowed to grow for 12 days in the light, after which the containers were placed in the dark and sediment added to two of the algae cultures, in order to simulate conditions which would prevail in the field as the algae died and sank to the pond floors. The addition of sediment hastened decay of the algae in the dark. Since none of the containers were bacteria-free, both sediment-containing and sediment-free cultures presumably had increased bacterial activity as algal decay proceeded.

TABLE X

LONG-TERM EXPERIMENT I

UPTAKE OF URANIUM AND MOLYBDENUM BY SPIROGYRA

DAY		PPM U IN SOLUTION	PPM MO IN SOLUTION	PH
0	CONTROL	1.2 ± 0.5	1.8 ± 0.0	4.2
	ALGAE	0.9 ± 0.0	2.1 ± 0.8	7.2
1	CONTROL	1.7 ± 0.2	1.8 ± 0.0	4.2
	ALGAE	0.9 ± 0.5	2.0 ± 0.3	7.2
3	CONTROL	2.1 ± 0.1	2.0 ± 0.0	4.0
	ALGAE	1.6 ± 0.3	2.3 ± 0.3	8.3
5	CONTROL	1.6 ± 0.1	2.0 ± 0.1	4.3
	ALGAE	1.1 ± 0.5	1.6 ± 0.1	6.9
7	CONTROL	3.5 ± 0.7	1.9 ± 0.0	4.0
	ALGAE	0.4 ± 0.0	1.3 ± 0.5	7.5
14	CONTROL	2.8 ± 0.2	2.3 ± 0.0	4.1
	ALGAE	0.5 ± 0.1	1.2 ± 0.2	7.8

The sediments became black, which is indicative of bacterial sulfate reducing activity. After 23 days in the dark (day 35), the blackening was most prominent around the decaying algae and present otherwise only below the surface in the sediment.

The addition of sediment in control containers increased the uranium in solution but not the molybdenum (Table XI). Levels of uranium were consistently higher in Chara containers than in either control condition. It is possible that the presence of the algae prevented precipitation of uranium which occured in control containers. After day 35, uranium levels dropped in algae cultures both with and without sediment (Table XI). In Chara only containers, the uranium concentration dropped from 14.5 ppm at day 35 to 4.8 ppm at day 154. The addition of sediment increased the extent of removal of uranium and also hastened the rate of removal, with levels dropping from 11.9 ppm at day 13 to 1.7 ppm at day 154. The cultures of Chara with sediment were the only condition in which molybdenum levels were lowered, with a decrease from 3.0 ppm at day 13 to 1.0 ppm at day 154. The decrease of both uranium and molybdenum in the Chara plus sediment cultures seemed to have reached equilibrium between day 70 and day 154, and no further release of metals was observed.

Experiment III (Table XII) repeated the conditions of Experiment II but used Shen's medium, which did not precipitate uranium to the degree observed in Bristol's medium.

TABLE XI

LONG-TERM EXPERIMENT II

UPTAKE OF URANIUM AND MOLYBDENUM

BY CHARA IN PRESENCE OF SEDIMENT

		PPM IN SOLUTION			
DAY		CONTROL		CHARA	
0	URANIUM	9.4±2.3		10.2±4.1	
	MOLYBDENUM	2.7±0.1		2.6±0.1	
	PH	7.7		7.7	
4	URANIUM	1.2±0.7		8.4±0.9	
	MOLYBDENUM	2.7±0.1		2.6±0.1	
	PH	7.1		7.8	
8	URANIUM	1.1±0.5		15.9±7.1	
	MOLYBDENUM	2.7±0.2		2.8±0.2	
	PH	7.1		7.7	
12	URANIUM	2.1±0.8		14.4±1.5	
	MOLYBDENUM	2.7±0.1		2.8±0.1	
	PH	7.0		7.9	
			SEDIMENT ADDED		SEDIMENT ADDED
13	URANIUM	2.5±0.8	1.0±0.1	13.2±0.4	11.9±0.3
	MOLYBDENUM	2.6±0.0	2.8±0.1	2.8±0.1	3.0±0.0
	PH	7.0	7.6	7.6	7.6
21	URANIUM	0.6±0.2	2.4±1.2	13.1±1.2	6.7±0.7
	MOLYBDENUM	2.8±0.0	3.5±0.0	2.7±0.0	2.7±0.0
	PH	7.0	7.8	7.5	7.5
35	URANIUM	0.9±0.3	6.6±1.3	14.5±1.8	4.0±0.5
	MOLYBDENUM	2.8±0.2	2.9±0.1	2.8±0.1	1.9±0.0
	PH	6.9	8.0	7.4	7.7

TABLE XI--continued

LONG-TERM EXPERIMENT II

UPTAKE OF URANIUM AND MOLYBDENUM

BY CHARA IN PRESENCE OF SEDIMENT

DAY		PPM IN SOLUTION			
		CONTROL		CHARA	
			SEDIMENT ADDED		SEDIMENT ADDED
70	URANIUM	0.2±0.0	8.3±2.2	4.1±0.2	1.9±0.4
	MOLYBDENUM	3.4±0.1	2.9±0.2	3.1±0.2	0.8±0.0
	PH	7.1	8.2	7.8	8.2
154	URANIUM	0.1±0.0	3.8±1.8	4.8 -	1.7±0.0
	MOLYBDENUM	3.2±0.4	3.2±0.1	3.2±0.1	1.0±0.0
	PH	7.1	8.1	8.1	8.1

The Spirogyra cultures contained some Oscillatoria, which lysed within 1-4 days, giving the medium a faint brown color. The Chara cultures showed new growth throughout the 13-day light period, while the Spirogyra cultures were decaying after 13 days. The Spirogyra containers had developed a surface growth of blue-green algae, predominately Lyngbya, by the end of the light period. By day 21, the control sediments without algae had a light, oxidized surface layer on the sediment, while the sediments with algae remained black throughout. After 21 days in the Spirogyra cultures and after 49 days in the Chara cultures, the blackening of the sediment surface and the presence of intact algae cells were no longer evident.

Removal of uranium was most pronounced in the Spirogyra cultures (Table XII). While the presence of sediment did not enhance the removal of uranium from solution in comparison to the sediment control, the algae increased the sediment's ability to retain uranium. At day 166, 2.1 ppm uranium remained in solution in the Spirogyra-sediment cultures and 3.8 ppm in the sediment control.

In the Chara cultures, growing algae had no effect on uranium removal, and some release of uranium from Chara seemed to have occured when the containers were transferred to the dark (Table XII, days 0-14). However, as Chara decayed in the presence of sediment, levels of uranium fell from 8.0 ppm at day 14 to 2.0 ppm at day 49. Lessened

TABLE XII

LONG-TERM EXPERIMENT III

UPTAKE OF URANIUM AND MOLYBDENUM

BY CHARA AND SPIROGYRA IN PRESENCE OF SEDIMENT

DAY		PPM IN SOLUTION CONTROL	CHARA		SPIROGYRA	
0	URANIUM	5.8±1.9	5.8±0.8		5.3±2.1	
	MOLYBDENUM	2.2±0.3	1.9±0.2		2.3±0.2	
	PH	7.4	7.8		7.8	
3	URANIUM	5.1±2.2	6.7±0.9		4.2±0.7	
	MOLYBDENUM	2.2±0.2	2.3±0.2		2.0±0.7	
	PH	7.4	7.7		7.8	
7	URANIUM	3.3±0.6	5.3±0.9		3.5±1.2	
	MOLYBDENUM	2.2±0.2	2.2±0.2		1.9±0.3	
	PH	7.4	8.0		7.9	
13	URANIUM	3.0±0.3	5.5±1.2		2.1±0.3	
	MOLYBDENUM	2.9±0.6	2.2±0.2		1.9±0.4	
	PH	7.4	8.0		7.7	
			SEDIMENT ADDED		SEDIMENT ADDED	SEDIMENT ADDED
14	URANIUM	4.3±0.5	6.0±1.5	7.8±0.1	8.0±1.0	3.8±0.8	3.5±0.8
	MOLYBDENUM	1.9±0.1	2.2±0.0	2.0±0.1	2.4±0.0	1.4±0.1	1.9±0.1
	PH	7.4	7.7	7.8	7.9	7.5	7.7

TABLE XII--continued

LONG-TERM EXPERIMENT III

UPTAKE OF URANIUM AND MOLYBDENUM

BY CHARA AND SPIROGYRA IN PRESENCE OF SEDIMENT

DAY		PPM IN SOLUTION					
		CONTROL	CHARA		SPIROGYRA		
			SEDIMENT ADDED		SEDIMENT ADDED		SEDIMENT ADDED
21	URANIUM	2.7±0.5	4.6±0.6	6.3±1.2	3.2±0.0	2.0±0.1	1.2±0.2
	MOLYBDENUM	2.4±0.0	2.0±0.5	2.5±0.0	2.3±0.0	2.5±0.0	1.0±0.1
	PH	7.4	8.1	7.6	7.8	7.7	7.6
49	URANIUM	3.7±0.1	5.2±0.0	6.5±0.3	2.0±0.0	1.7±0.6	1.5±0.2
	MOLYBDENUM	2.3±0.0	2.7±0.1	2.9±0.6	1.8±0.3	1.6±0.1	1.5±0.3
	PH	7.4	7.7	7.8	7.8	7.8	7.6
80	URANIUM	4.8±1.1	5.5±0.4	7.2±0.5	4.2±0.9	2.5±0.3	4.3±1.1
	MOLYBDENUM	2.1±0.0	2.8±0.1	2.3±0.1	1.8±0.5	2.2±0.3	2.7±0.2
	PH	7.6	7.6	7.6	7.6	6.0	7.6
166	URANIUM	4.8±0.7	3.8±0.5	5.5±0.2	3.8±1.1	0.6±0.0	2.1±0.5
	MOLYBDENUM	2.9±0.3	2.3±0.2	3.8±0.1	4.1±1.4	3.2±0.2	4.7±0.3
	PH	-	7.4	7.3	-	5.9	7.7

decay of the algae after day 49 allowed re-establishment of an oxidizing zone at the sediment surface, which coincided with partial remobilization of uranium. The similarity of day 80 and day 166 values, 4.2 ppm and 3.8 ppm respectively, suggests that an equilibrium had been established in the system.

No cultures in Experiment III showed irreversible removal of molybdenum, although Spirogyra cultures reduced molybdenum substantially by day 21, from 2.2 ppm to 1.4 ppm. However, molybdenum levels returned to original levels in solution in all cultures and were, in fact, higher than the initial values in the algae and sediment cultures.

Experiment IV (Table XIII) was intended to magnify the effect of decaying algal material on sediment retention of uranium and molybdenum. The amounts of sediment and algal material were increased (Table IV), and the algal material, dried Chara, was dead at the start of the experiment. By day 16, bacterially mediated decay was extremely vigorous in the algae cultures, which were heavily overgrown with a surface layer of bacteria. The algae plus sediment cultures were blackened throughout the solution and sediment, while the sediment alone cultures were blackened only beneath the surface of the sediment. High bacterial activity was evident by visible inspection even after 47 days. However, after day 74 (Table XIV), the algae cultures had lost the blackened appearance, and the surface masses of bacteria

TABLE XIII

LONG-TERM EXPERIMENT IV

UPTAKE OF URANIUM AND MOLYBDENUM BY CHARA AND SEDIMENT IN DARK-MAINTAINED CULTURES

DAY		PPM U*	PPM MO*	PH
2	SEDIMENT	24.6±0.1	6.3±0.5	
	ALGAE	15.3±2.5	7.3±0.6	
	SEDIMENT+ALGAE	12.8±0.5	4.2±0.2	
16	SEDIMENT	8.6±0.4	4.2±0.5	7.61
	ALGAE	7.2±0.3	2.6±0.2	7.31
	SEDIMENT+ALGAE	1.0±0.2	0.4±0.4	7.23
47	SEDIMENT	2.6±0.3	2.7±0.4	7.75
	ALGAE	4.1±1.1	3.1±0.1	7.50
	SEDIMENT+ALGAE	0.3 -	0.06±0.05	7.80
108	SEDIMENT	1.2±0.3	1.4±0.4	7.62
	ALGAE	3.9±1.2	3.0±0.0	7.72
	SEDIMENT+ALGAE	1.0±0.4	0.03±0.01	8.02
190	SEDIMENT	0.7±0.0	1.7±0.1	7.70
	ALGAE	1.1 -	3.2±0.3	7.79
	SEDIMENT+ALGAE	1.9±0.3	1.0±0.0	7.90

*All cultures were spiked at day 0 with 20 ppm U and 5 ppm Mo

were absent. In the sediment plus algae cultures, the algae and sediment surfaces were still blackened, but an oxidized layer had formed in the sediment alone. The Chara cells remained partially intact even at the end of the test, though most of the algal material had disintegrated into particles. Table XIV gives Eh values in the cultures for days 74, 108 and 190. Both water and sediment were reducing at day 74, but by day 108 only the sediment remained reducing.

The percentage removal of 20 ppm uranium and the 5 ppm molybdenum spikes which were added to all containers is given in Table XV. Initial release of uranium from sediment and from algae containers was seen at day 2. Sediment alone removed more uranium (94%) and molybdenum (72%) than algae alone (80% for uranium and 40% for molybdenum), but the combination of sediment plus algae increased removal of uranium to 95% and of molybdenum to 99%. Removal of both metals was essentially complete after 16 days in the algae plus sediment cultures, but some release of both occurred by day 190. This release was not observed in either the sediment alone or the algae alone.

TABLE XIV

EH MEASUREMENTS FOR LONG-TERM EXPERIMENT IV:
CHARA AND SEDIMENT IN DARK-MAINTAINED CULTURES

DAY	SEDIMENT		ALGAE	SEDIMENT+ALGAE	
	water	sediment	water	water	sediment
74	-204 ± 5	-502 ± 46	-415 ± 5	-302 ± 2	-515 ± 35
108	$+150\pm4$	-339 ± 63	$+107\pm2$	$+88\pm1$	-392 ± 23
190	$+68\pm1$	-382 ± 80	$+70\pm0$	$+50\pm25$	-393 ± 105

TABLE XV

LONG-TERM EXPERIMENT IV
PERCENTAGE OF URANIUM AND MOLYBDENUM REMOVED BY
CHARA AND SEDIMENT IN DARK-MAINTAINED CULTURES

DAY	SEDIMENT		ALGAE		SEDIMENT+ALGAE	
	U	MO	U	MO	U	MO
2	+23%	+26%*	23%	+46%	36%	16%
16	57%	16%	64%	48%	95%	92%
47	87%	46%	79%	38%	98%	99%
108	94%	72%	80%	40%	95%	99%
190	96%	66%	94%	36%	90%	80%

*Plus sign indicates a percentage increase over the amount of spike added at day 0; values otherwise represent a percentage decrease.

IV. DISCUSSION

The marked differences in accumulation of uranium and molybdenum by the different algae in the pond system may be due to the different chemistry of the two metals, but the roles of the metals in the algal life cycle may also play a part in determining their accumulation.

Molybdenum is probably present in the pond water as the anion MoO_4^{2-} (Bloomfield and Kelso, 1973), and its accumulation by direct adsorption onto negatively charged algal cell surfaces seems unlikely. Bloomfield and Kelso (1973), however, do point to the suggestion of Szalay (1969, Arkiv. Mineralogi Geologi 5: 23-36) that molybdenum is reduced by organic matter to exchangeable cationic forms, so that adsorption of molybdenum may occur in algae.

Molybdenum can be an essential trace element for green algae, and its requirement for those blue-green algae which fix nitrogen has been demonstrated (O'Kelly, 1974). It is possible that cells requiring molybdenum would have mechanisms to regulate levels of the metal in the cell interior. Chara in particular shows little variation in molybdenum levels. While a regulatory system for molybdenum has not been demonstrated in Chara, Robinson (1969) has found active uptake of sulfate ions into the vacuole of Chara australis by a pump across the plasmalemma, which might provide a pathway for uptake of MoO_4^{2-} as well. In

the field-collected algae, levels of molybdenum varied 10-fold over different collections, which may be due to adsorption onto cells in addition to uptake into the cells. The ability of Spirogyra to adsorb molybdenum on a short-term basis was indicated by the 24-hour uptake experiments, where an increase in surface area led to an increase in removal of molybdenum from solution. However, the different levels in the collections may also be due to varying populations of algae over the year, especially since the winter collection, which showed higher molybdenum content, also contained a higher proportion of the blue-green Oscillatoria.

In contrast to molybdenum, uranium is generally considered an abiological element (Taylor, 1979), and its uptake is more likely to be governed by physical-chemical factors than by active cellular processes. The uranyl ion has an extremely high affinity for organic material (Jackson, Jonasson and Skippen, 1978), and adsorption onto the negatively charged algal cell surfaces probably accounts for the high levels of uranium in the pond algae. Chara shows a lower affinity for uranium than the filamentous algae. In a study of radionuclide accumulation by Chara species, Marchyulenine (1978) felt that the presence of high levels of calcium carbonate compounds in the cell wall reduced the number of cation exchange sites in Chara compared to other algae. Sikes (1978) noted that in another

calcium-accumulating alga, Cladophora, calcium carbonate increased with the age of the cells, and this calcium was not subject to appreciable ion exchange.

The seasonal variation in both uranium and molybdenum levels in the filamentous algae suggests that adsorptive processes are important in the accumulation of the metals in the algae cells, since extent of adsorption depends not only on the amount of metal available per unit of surface area but also on the length of exposure of the surface to the metal. Thus, longer-lived cells would be exposed to more metals than short-lived cells. The September collections represent quickly growing populations, while the January collections were taken from sparser populations which, due to the slower growth rate in winter, had a higher turnover time than the summer populations. April may represent a transition from chiefly perennial populations to annual summer blooms of algae. Knauer and Martin (1973) found that levels of heavy metals in marine phytoplankton were low during periods of algal blooms due to dilution of the amount of metal available per unit mass of phytoplankton. While the algae ponds in this study did support denser populations in the summer, the increase in cell number probably was not great enough to alter the ratio of metal to algae. More important is the increased length of exposure time for the winter populations. Hodge, Koide and Goldberg (1979) found that marine macroalgae adsorbed

uranium, plutonium, and polonium in direct relationship to exposure time. The longer exposure could also lead to exchange absorption into the cell interior, as described in the long-term exposure of Cladophora to lead (Gale and Wixson, 1979).

The results of the 24-hour uptake experiments support the field evidence for different interactions between the metals and the different algae. Short-term uptake was not observed in Chara, which in fact accumulated both metals at much lower levels than the filamentous algae in the field. In the Spirogyra 24-hour test, the cell material showed a limited capacity to adsorb molybdenum, while uranium uptake increased in higher external concentrations. The disruption of the cells to form particulate suspensions evidently increased surface area for binding, although release of cellular constituents, which may form insoluble complexes, might also have been a factor (Ferguson and Bubela, 1974). Horikoshi et al. (1979) also observed an increase in adsorption of uranium in disrupted cells. In an 8 ppm solution of uranium, adsorption increased from 15,600 ppm in living Chlorella to 67,200 ppm in heat-killed cells.

The long-term laboratory studies indicate that the pond algae, in the form of particulate, decaying material, can be instrumental in removing metals from solution. However, the patterns of retention and release of uranium and molybdenum as the algae decayed in the presence of sediment indicate that maintenance of reducing conditions in the

sediment or in the algal cultures is critical to the sequestering of the metals. In the model systems, the period of algal decomposition was associated with increased bacterial activity and an enhancement of reducing conditions in the sediment-containing cultures. The algae cultures without sediment probably decomposed mainly in aerobic conditions, except in Experiment IV, where the high proportion of algal material led to reducing conditions in the first two months of incubation. When sulfate levels are high, as they are in the pond water, anaerobic decomposition of algae is accompanied by sulfate reduction even in the absence of sediment (Foree and McCarty, 1970). Decomposition was associated in the model systems with removal of molybdenum and uranium from solution, but the period following decompostion was characterized by the partial to complete release of the metals back into solution. The release of molybdenum was surprising, since sulfides which are formed during sulfate reduction can form highly insoluble molybdenum disulfide. It is possible that molybdenum was precipitated in the reduced Mo(IV) state but did not form the sulfide mineral, so that release occurred as molybdenum was re-oxidized. However, molybdenum showed little or no release in Experiment IV, although oxidizing conditions were established in the water compartment and at the sediment surface for at least the last 82 days of the experiment.

In most cases, the addition of algae to sediment increased the sediment's ability to retain uranium. Reduction of uranium to the insoluble U(IV) ion may have contributed to the removal, with adsorption onto the organic material another possibility. Taylor (1979) has pointed out that in sediments, the production of hydrogen sulfide by sulfate-reducing bacteria may increase the adsorption of uranyl ion onto organic material present in the sediment, since iron and other sulfide-forming metals could be desorbed from the organic material to increase adsorption sites for uranyl ion.

The implication from the laboratory studies is that, while the algae are instrumental in removing metals from solution, the process is reversible unless the system contains substantial organic material. Also, while organic material accelerates the rate of removal and sometimes the extent of removal from water, retention of the metals in sediments is also reversible unless the system contains a high volume of sediment. These conclusions point to major problems in improving the existing pond system in respect to removal of uranium and molybdenum.

In the present system, uranium levels are reduced significantly between the initial influent from the mines and the final effluent, but the removal is evidently effected almost entirely by the ion exchange system in section 35, and possibly by precipitation in the large

section 36 settling pond. Since the rate of flow from both sections 35 and 36 is about the same ($2x10^6$ gallons day^{-1}), the average input of uranium into the system as a whole can be calculated as the average of the two sections, which is 4.0 ppm uranium (see Table I for pond water values). The reduction to 0.7 ppm in the final effluent is significant at the .01 level. However, as can be inferred from the similar averages of uranium concentrations in the three algae ponds, there is no significant reduction of uranium in solution in any of the algae ponds or indeed between the influent to the algae ponds and the final effluent.

The case of molybdenum is similar. There is no significant reduction of molybdenum in solution throughout the system; the values in the algae ponds are lower than in section 35 because of mixing with the waters from section 36, which have very low levels of dissolved molybdenum. Although the sediments concentrate both metals susbstantially over concentrations in water, the amount of metals removed is not substantial enough to be reflected in the water concentrations.

Part of the problem in seeing statistically significant trends in the data is due to wide fluctuations over the samples values, as indicated by the relatively high standard deviations in Table I. More data might overcome part of this problem, if in fact there are trends which are being masked by the present sampling regime. However,

it is important to remember the volume of water which passes through the system yearly. At a combined inflow of $4x10^6$ gallons a day, or $15x10^6$ liters a day, the pond system receives $5.5x10^9$ liters every year. Based on the uranium and molybdenum values in the influent to the algae ponds of 0.9 ppm for each metal, this volume amounts to $4.95x10^9$ mg (4.95 metric tons) each of molybdenum and uranium entering the algae ponds yearly. The filamentous algae present in the ponds now accumulate an average of 2000 ppm uranium and 130 ppm molybdenum on a dry weight basis. Given this level of removal, it would take $4.95x10^9$ mg U x (10^3 g algae/2000 mg U) = $2.5x10^9$ g dry weight algae produced per year to remove the uranium entering the algae ponds. If the ponds were fertilized to increase productivity, could this amount of algae be grown in the existing pond system? Wetzel (1966) found that a hypereutrophic lake produced 570 g C m^{-2} yr^{-1}. Using a common value found for carbon content in algae of 40% of dry weight (Ferguson and Bubela, 1974), 570 g C corresponds to a dry weight of algae of 1425 g m^{-2} yr^{-1}. In order to grow $2.5x10^9$ g algae yr^{-1}, the amount needed to accumulate the influent uranium, the pond system would have to be expanded to $2.5x10^9$g x (m^2/1425 g) = $1.75x10^6$ m^2, or 17.5 hectares. The ponds now cover 0.15 hectares. Obviously, relying on algal removal alone will not answer the problem of improving the pond system.

Increasing pond productivity may improve removal

indirectly, as suggested by the long-term laboratory studies. The ponds are fairly unproductive now, which is typical of the early to middle stages of succession in Chara ponds, where phosphate levels are quite low (Crawford, 1979). Later stages of Chara succession are characterized by overgrowth of planktonic algae and higher plants, which increase pond productivity. Additions of phosphate and nitrogen-containing fertilizers would accelerate the replacement of Chara with more productive annual green and blue-green planktonic algae. Promoting eutrophication of the pond system would increase the amount of organic substrate available for sulfate-reducing bacteria and would promote reducing conditions in the pond sediments. Laboratory experimentation has indicated that enhancement of growth of Desulfovibrio and Desulfotomaculum and thereby of sulfate reduction by these bacteria in sediments resulted in removal of uranium, molybdenum and selenium from solution (Brierley and Brierley, 1980). It is possible that, while the algae cannot function alone to remove excess trace contaminants, the effect of increased algal productivity on water and sediment chemistry might be significant.

APPENDIX A
ANALYTICAL PROCEDURES

A. The following procedure for uranium analysis is taken from the method of Johnson and Florence (1971).

Uranyl ion can be separated from contaminants by solvent extraction using trioctylphosphine oxide in cyclohexane. A yellow colored complex of uranium (VI) dibenzoylmethane forms instantly by introducing an aloquot of the extract into a pyridine-ethanol solution of dibenzoylmethane. The analysis has a sensitivity of 0.05 ppm uranium.

Reagents

1M hydroxylamine sulfate:	Dissolve 164.14 g of hydroxylamine sulfate in 1 liter of distilled water.
1M potassium flouride:	Dissolve 58.1 g of KF in 1 liter of distilled water.
0.05M trioctylphosphine oxide in cyclohexane:	Dissolve 19.3 g of trioctylphosphine oxide in 1 liter of cyclohexane.
50% pyridine in ethanol:	Mix 1:1 pyridine in ethanol.
1% mixed color reagent:	Dissolve 10 g of dibenzoylmethane in 1 liter of absolute ethanol.
uranium standard:	Dissolve 1.0000 g of pure U_3O_8 in a minimum amount of nitric acid and dilute to 1 liter with distilled water.
working standard:	Dilute 1 ml of 1000 ppm standard to 100 ml with distilled water. Make fresh daily.

Procedure

1. Prepare blank and standards of 0.1, 0.2, 0.3 and 0.5 ppm U_3O_8 using the working standard of 10 ppm.
2. Dilute blank, standards and samples to 100 ml in 250 ml separatory funnels.

3. Add 2 ml of concentrated HNO_3.
4. Add 1 ml of hydroxylamine sulfate solution to reduce iron.
5. Add 1 ml of KF solution to complex molybdenum, zirconium and titanium.
6. Add 5 ml of TOPO in cyclohexane.
7. Stopper the funnel, shake gently, relieve internal pressure, and shake vigorously for 2 minutes.
8. Allow sample to stand until phases separate, about 5 minutes.
9. Drain off and discard bottom aqueous layer.
10. With a dry pipet, remove 2.0 ml of the organic phase and transfer to dry 10 ml volumetric flask.
11. Add approximately 1 ml of the 50% pyridine-ethanol solution to the sample until a clear solution is obtained.
12. Add 2.0 ml of the mixed color reagent.
13. Bring to volume with the 50% pyridine-ethanol solution and mix well.
14. Allow the sample to stand for at least 5 minutes for color development.
15. Set spectrophotometer to zero absorbance with the blank at 405 nm. Read absorbance of standards and samples.
16. Prepare standard curve and calculate the concentrations of the samples.

B. The following molybdenum analysis is taken from Meglen and Glaze (1973).

Molybdenum is analysized colorimetrically as the thiocyanate-molybdenum complex. The analysis has a sensitivity of about 5 ppb molybdenum.

Reagents

1% ferrous ammonium sulfate:	Dissolve 1 g $Fe(NH_4SO_4)_2$ in 100 ml distilled water with 1 ml concentrated sulfuric acid.
10% potassium thiocyanate:	Dissolve 5 g KSCN in 45 ml of distilled water. Make fresh daily.
10% stannous chloride:	Dissolve 100 g $SnCl_2$ in 125 ml concentrated nitric acid, heat to dissolve. Add slowly to 900 ml distilled water. Store in container with piece of pure tin metal.

Procedure

1. Prepare blank and standards of 50, 100, 300 and 500 ppb Mo in 250 ml separatory funnels.
2. Dilute blank, standards and samples to 100 ml.
3. Add 2 ml of concentrated HCl.
4. Add approximately 0.2 g sodium tartarate to complex tungsten.
5. Add 0.5 ml of 1% ferrous ammonium sulfate.
6. Add 3.0 ml of KSCN solution.
7. Allow sample to stand 15 minutes to develop pink to red color.
8. Add 9.0 ml of 10% stannous chloride solution.
9. Allow sample to stand 15 minutes. Pale amber thiocyanate-molybdenum complex will form.
10. Add 10.0 ml of iso-amyl alcohol. Shake for 1 minute.
11. Allow phases to separate, about 15 minutes. Drain off and discard bottom aqueous layer.
12. Using dry pipet, remove aliquot of organic phase and transfer to spectrophotometer tube.
13. Set spectrophotometer to zero absorbance with the blank at 465 nm. Read absorbance of standards and samples.
14. Prepare standard curve and calculate the concentrations of the samples.

APPENDIX B

STATISTICAL TEST VALUES

One-way analyses of variance were applied to data collected over the study period in order to determine whether differences between ponds or between algae collections were statistically significant. In tests 1-7 in the table below, different pond sites were compared. Thus, the classes are collection sites, as described in Table I, and the values within a class are the monthly sample levels. In tests 8 and 9, the metal levels in the three algae collections were compared, with the levels from different sample sites (described in Table VII) constituting the values within a class. Tests were considered significant if the calculated F value was greater than the F value at P=0.01 (taken from Snedecor and Cochran, 1967).

DEGREES OF FREEDOM		F VALUE	CALCULATED	DESCRIPTION OF TEST
Between Classes	Within Classes	P=0.01	F VALUE	
1	16	8.53	10.00	Uranium: combined minewaters section 35 and 36 compared to effluent algae pond 3
1	16	8.53	10.46	Uranium: combined minewaters section 35 and 36 compared to influent algae pond 1
3	20	4.94	0.93	Uranium: comparison of ponds in section 35 among themselves
1	12	9.33	3.36	Uranium: minewater section 36 compared to sec. 36 pond B-2 effluent
3	20	4.94	0.54	Uranium: algae ponds 1, 2 and 3 compared among themselves

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RADIONUCLIDES IN NEVADA TEST SITE GROUNDWATER: TRANSPORT BY CLAY COLLOIDS

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The Cheshire (U20n) event was a nuclear detonation fired February 14, 1976, at the Nevada Test Site (NTS). It was detonated in a fractured rhyolitic lava formation 1167 m below the surface and 537 m below the water table. Yield was in the 200 to 500 kiloton range. A slant-drilled re-entry hole was cased and perforated within the cavity. Cavity water was intermittently pumped and sampled between September 1983 and May 1985. Thereafter, the well was plugged at 860 m and pumping was resumed from new perforations in the 763-858 m depth range. Because of the slant of the hole the new location was estimated to be approximately 300 m above the cavity and displaced approximately 100 m hydrologically down-gradient.

Because a number of strongly sorbing or insoluble radionuclides were observed in the "dissolved" phase (0.45 μm filtered) of early cavity samples, Lawrence Livermore National Laboratory initiated a series of filtration and ultrafiltration experiments using large-volume samples. Filtrates and ultrafiltrates were dried, weighed, gamma counted and further characterized; filters were gamma counted. Table 1 presents the distribution of masses between the dissolved phase and various particulate size ranges based on weight relationships.

Table I: Dissolved and Colloidal Material in Cheshire Samples[a]

Sample	TDS (mg/L)[b]	Colloid Size range (μm)	Est. Colloid Mass Con. (mg/L)
B-1 (cavity)	256	>0.006	55
B-2 (cavity)	(256)	(0.45 - 0.006)	(63)
B-3 (cavity)	(263)	(0.2 - 0.003)	(35)
		(0.2 - 0.05)	(25)
B-4 (cavity)	263	0.05 - 0.003	10.1
B-5 (external)	216	0.05 - 0.003	4.6
B-6 (external)	218	0.05 - 0.003	4.3

Notes: [a]values in parentheses calculated for non-ultrafiltered samples by assuming TDS for other samples applied.
[b]calculated from weight of salts recovered from dried ultrafiltrate.

X-ray diffraction studies of ultrafiltrate and retentate solids showed that the ultrafiltrates were composed of evaporite salts (halite, trona, aragonite), but that the retentates were dominated by quartz and (Ca, K) feldspars. The equilibrium geochemical model code EQ6 was run for a variety of scenarios using both laboratory and field pH and temperature observations and water chemistry determined by analysis

of 0.45 μm filtered samples. All runs predicted the presence of a solid phase dominated by quartz (50 - 60 mg/L) and containing 1-10 mg/L muscovite and phengite (field temperature and pH) or kaolinite and montronite-Ca (lab temperature and pH). Although chemical equilibrium is not necessarily a valid guide to actual conditions, both the mass ranges and the types of minerals predicted are in qualitative agreement with the observations.

X-ray fluorescence results for the ultrafiltrate and retentate from sample B-4 are presented in Table 2. Since the actual mass in the 0.05-0.003 μm particle size range is 3.0% of the TDS, the elements K, Fe, Mn, Rb, Pb, Cs, Ba, La and Ce are quite concentrated in the colloidal phase. The potassium effect is ascribable to the potassium-bearing clay minerals in the solids, while the other elements are among those whose sorption or solubility characteristics make them logical candidates for particulate association.

Table II: X-ray Fluorescence Analysis, Sample B-4

Element	Salts (ppm)	Retentates ppm	Calc. Colloidal Fract. (0.05-0.003 μm)
K	1800	25400	0.356
Ca	2900	2200	0.028
Mn	96	540	0.180
Fe	318	5470	0.403
Ni	32	20	0.023
Cu	159	280	0.068
Zn	1980	610	0.011
As	49	22	0.016
Br	140	19	0.004
Rb	19	190	0.281
Mo	41	6	0.004
Pb	35	320	0.263
Cs	<5	14	>0.108
Ba	25	203	0.241
La	<5	19	>0.129
Ce	<5	84	>0.397

Table 3 presents data on the fraction of the total activity passing the final filter (or ultrafilter) for those nuclides observed in all or most of the samples. Total concentrations of the more soluble nuclides (Sb, Cs) were only a factor of two (or less) lower at the external location than in the cavity, but total concentrations of Mn and Co radionuclides were reduced by factors of 4-5 or more and concentrations of the lanthanide nuclides were more than an order of magnitude lower outside of the cavity.

Table 3 shows very significant differences between the ultrafiltered and the conventionally filtered cavity samples, and somewhat subtler differences between the ultrafiltered cavity and external samples. We also note that the behaviors of different isotopes of the same element are generally quite similar.

We can summarize the following major conclusions from these observations:

1. Essentially all of the transition element and lanthanide nuclides observed are associated with particles, the bulk of which are not filterable by standard filtration techniques.

2. The presence of these nuclides outside of the cavity, albeit at concentrations greatly reduced relative to more conservative tracers, strongly suggests that they are moving by particle transport.

3. The available chemical, mineralogical and geochemical data strongly suggest that the particles involved are colloidal clays which serve as a substrate for the transport of sorbed nuclides.

Table III: Fraction of Activity Passing Final Filter

Sample:	B1	B2	B3	B4	B5	B6
Filter size (μm):	(Cavity) 0.006	(Cavity) 0.45	(Cavity) 0.2	(Cavity) 0.003	(External) 0.003	(External) 0.003
Nuclide						
^{22}Na	0.95	0.98	0.97	0.89	0.95	~1.00
^{40}K	0.33	0.76	0.97	0.31	0.73	0.83
^{54}Mn	$<1.6\times10^{-4}$	0.73	0.87	$<2.4\times10^{-4}$	7×10^{-5}	n.d.
^{60}Co	$<1.3\times10^{-3}$	0.68	0.89	0.02	<0.01	<0.01
^{106}Ru	0.09	0.83	0.93	0.17	0.24	0.29
^{125}Sb	0.95	0.99	0.99	0.97	0.99	0.99
^{134}Cs	0.59	0.84	0.98	0.70	0.95	~1.00
^{137}Cs	0.56	0.80	0.98	0.68	0.89	0.89
^{144}Ce	<0.01	0.76	0.92	<0.05	<0.22	n.d.
^{152}Eu	$<3.5\times10^{-3}$	0.80	0.92	0.01	<0.03	<0.11
^{154}Eu	$<4.6\times10^{-3}$	0.79	0.92	<0.01	<0.03	<0.04
^{155}Eu	$<3.6\times10^{-3}$	0.80	0.91	<0.01	<0.09	<0.05

Notes: n.d. = not detected

[1]Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract No. W-7405-ENG-48.

BIODEGRADATION OF DRILLING FLUIDS: EFFECTS ON WATER CHEMISTRY AND ACTINIDE SORPTION

by

Larry E. Hersman

ABSTRACT

Several million gallons of drilling fluids have been introduced into, or immediately adjacent to, the candidate high-level nuclear waste repository at Yucca Mountain, Nevada. Studies conducted by the Nevada Nuclear Waste Storage Investigations (NNWSI) Project demonstrate that these drilling fluids are biodegradable by a variety of micro-organisms. Further studies demonstrate that one species of bacteria is able to strongly sorb $^{239}Pu^{4+}$.

I. INTRODUCTION

Several million gallons of drilling fluids were used during characterization of the geology and hydrology of the site for a candidate high-level nuclear waste repository at Yucca Mountain, Nevada. This activity was performed by engineers involved in the Nevada Nuclear Waste Storage Investigations (NNWSI) Project. The drilling logs from wells on the site indicate that a significant amount of the fluids was lost in the Topopah Spring Member, the geologic location of the candidate repository. The Topopah Spring Member has been characterized as a welded tuff with low permeability and high fracture content. Montazer and Wilson[1] have suggested that in this member lateral movement may exceed downward rates, a statement substantiated by the recent discovery of drilling fluids in the USW UZ-1 hole (air drilled without fluids). Interestingly, UZ-1 is located several hundred meters upgradient from the closest fluid-drilled holes (USW G-1 and H-1) and its drilling was limited to the unsaturated zone, at a depth approximately equal to that of the repository.

In addition to those drilling fluids, contractors plan to introduce several other forms of organic materials into the block candidate site. During the construction of the Exploratory Shaft, items such as diesel fuels and exhausts, hydraulic fluids, and additional drilling fluids will be routinely discarded. Investigators can also anticipate that many of the same materials will be used during the construction of the actual repository. Therefore, before the repository is completely sealed, significant amounts of organic materials will be located near it.

Our specific concern is that these organic materials may be used as growth substrates by large numbers of microorganisms, which in turn may influence the transport of radioactive elements from the repository. Microorganisms can affect transport in one or more of the following ways:

1) alter the composition of the groundwater chemistry through changes in pH or Eh,
2) solubilize radioactive elements by producing chelating agents,
3) transport the radionuclide by biological movement,
4) transport the radionuclide by colloidal dispersion, or
5) retard the transport of the radionuclide by sorption onto a non-motile solid phase.

The microbiological research being performed as part of the NNWSI Project can be divided into three sequential research tasks. First, we must determine the susceptibility of drilling fluids and other exogenous materials to microorganisms indigenous to Yucca Mountain. Second, we must ascertain the potential for sorption of radioactive elements by these biodegrading organisms. Finally, we need to document the effect of microorganisms on transport. The results of the first and second tasks are presented in this paper, and research on the third task has recently been initiated.

As stated earlier, during the exploration of Yucca Mountain as a possible location for the candidate high-level nuclear waste repository, several million gallons of drilling fluids were lost. Table I contains a list of the drilling fluids lost in wells located within a 3-km radius of the G-4 well, the proposed location of the Exploratory Shaft.

TABLE I

LOCATION AND VOLUMES OF DRILLING FLUIDS LOST NEAR WELL G-4

Well	Location	Quantity	Characteristics
USW H-1	Drill Hole Wash (DHW)	582,710 gal	Detergent/Water 1:153
USW H-3	Block	322,325 gal	D/W 1:60
USW H-4	Block	418,117 gal	D/W 1:325
USW H-5	Block	711,782 gal	D/W 1:141
USW H-6	West of Block	931,811 gal	D/W
USW G-1	DHW	2,600,000 gal	Polymer
USW G-4	Block	not available	Polymer
UE-25a#1	DHW	not available	Polymer
UE-25a#4	DHW	not available	Polymer
UE-25a#5	DHW	not available	Polymer
UE-25a#6	DHW	not available	Polymer
UE-25a#7	DHW	not available	Polymer
UE-25bl H	DHW	1,282,280 gal	D/W 1:300
UE-25wt#4	2 km N.E. of G-4	not available	D/W
USW wt#4	Block	not available	D/W

The average number of gallons of drilling fluid lost was 978,432. This total does not include diesel fuels or lithium chloride fluids. Based on the data available for seven drill holes, we estimate that nearly 15 million gallons of fluids have been introduced into this area (978,432 x 15 = 14,676,482). The drilling fluids lost were either ASP-700 (Nalco Chemical Company), a polymer-based fluid used in geologic test holes, or 5622 (Turco Chemical Company), a detergent-based fluid used in the drilling of hydrology wells. The following is a list of the ingredients in each fluid:

1. Turco 5622 (Detergent)
   77–87% Water
   10–20% Isopropyl alcohol
   <0.5% Gelatin
   <0.5% Ammonia
   <0.5% Sodium nitrite
   2.0% Surfactant (linear dodecylbenzene sulfonic acid)
2. Nalco ASP–700 (Polymer)
   43.2% Water
   29.0% Polymer (acrylamide co-poly w/Na acrylate)
   27.8% Light hydrocarbon
   a) Parafinic solvents (hexane, pentane) – 91%
   b) Emulsifier (or polyethylene glycol p-isooctylphenyl ether) – 7.2%
   c) EDTA (ethylenediaminetetraacetic acid) – 1.8%

Both Turco 5622 and ASP–700 contain oxidizable energy sources. For the Turco 5622 isopropyl alcohol, gelatin and the surfactant are biodegradable. For the ASP–700, most of the light hydrocarbons can serve as energy sources. It is also important to emphasize that water from any of the Yucca Mountain wells contains salts necessary for microbiological growth. Table II was taken from the NNWSI report, "Groundwater Chemistry Along Flow Paths Between a Proposed Repository Site and the Accessible Environment."[2] In comparison to the following list of constituents added to a minimal salts bacteriological medium, we see that there are ample salts in the groundwater at Yucca Mountain to support microorganisms:

Salts	$mg \cdot L^{-1}$
$NaNO_3$	112.0
K_2HPO_4	37.5
KH_2PO_4	75.0
$NaSO_4 \cdot 7H_2O$	37.5
$NaCO_3$	20.0
$CaCl_2$	25.0
$NaSiO_3 \cdot H_2O$	58.0
pH	7.0

TABLE II

ELEMENTAL AND ANION CONCENTRATIONS IN GROUNDWATERS
FROM THE VICINITY OF YUCCA MOUNTAIN

Field Well	Concentration (mg/l) pH	Ca	Mg	Na	K	Fe	Mn	Cl^-	SO_4^{2-}	HCO_3^-	NO_2^-	NO_3^-
USW VH-1	7.5	10	1.5	80	1.9			10	45	165		
USW H-6	7.4	5.5	0.22	74	2.1	0.12	0.04	7.7	27.5		N.D.	5.3
USW H-3	9.4	0.8	0.01	124	1.5	0.13	0.01	8.3	31.2		<0.10	0.2
USW H-5	7.1	1.1	0.03	54	2.3	0.01	N.D.	5.7	14.6		N.D.	8.6
USW G-4	7.1	9.2	0.15	56	2.5	0.04	0.02	5.5	15.7		N.D.	5.5
USW H-1	7.5	6.2	<0.1	51	1.6			5.8	19	122		
USW H-4	7.4	10.8	0.19	84	2.6	0.03	0.005	6.2	23.9		N.D.	4.7
UE-25b#1	7.7	19.7	0.68	56	3.3	0.04	0.004	7.1	20.6		N.D.	0.6
UE-25b#1	7.2	18.4	0.68	46	2.5	0.69	0.36	9.8	21.0		0.5	2.2
UE-25b#1	7.3	17.9	0.66	27	3.0	0.08	0.07	6.6	20.3		N.D.	4.5
J-13	6.9	11.5	1.76	45	5.3	0.04	0.001	6.4	18.1		N.D.	10.1
UE-29#2	7.0	11.1	0.34	51	1.2	0.05	0.03	8.3	22.7		N.D.	18.7
J-12	7.1	14	2.1	38	5.1			7.3	22	119		
UE-25p#1	6.7	87.8	31.9	171	13.4	<0.1	<0.1	37	129		N.D.	<0.1

An inventory of organic materials that will be used during the construction of the Exploratory Shaft at Yucca Mountain is listed below. The inventory was prepared by V. Gong of Reynolds Electrical & Engineering Co., Inc.

- Diesel fuel
- ATF
- Gear oil
- Rock drill oil/vegetable base
- Paints
- Dust suppressant*
- $CaCl_2$ tire ballast
- Brake fluid
- Engine oil
- Greases, including wire rope
- Cleaning solvents
- Antifreeze*
- Road oil (chip seal)*
- Service water tracers used for the following processes:
  - Drilling* - face, rockbolt, corehole, borehole
  - Wetdown - muckpiles, dust control
  - Drift-wall cleaning for geologists
  - Structural backfill compaction
  - Water-line leaks
  - Scrubber cleanout water*
- Concrete (water, sand, cement, aggregate grout, curing and form release chemicals, shotcrete, and additives)
- Human fluid waste
- Fire extinguishing chemicals
- Explosives and explosive residue
- Drilling fluids - ES2 pilot hole.

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*Fluids or chemicals that may have reasonable alternatives.

Table III presents an approximation of the volume of fluid to be used during the Exploratory Shaft construction.

TABLE III

ESTIMATE OF FLUID EXPOSURE DURING EXPLORATORY SHAFT CONSTRUCTION

Phase Description	Duration (Weeks)	Hydraulic Oil (gal)		Grease (lbs)		Anti-Freeze (gal)	Water		Rock Drill Oil	
		Surf	U.G.	Surf	U.G.	Surf	Surf	U.G.	Surf	U.G.
1. Surface Facility Construction										
Site Preparation	14	225	—	293	—	84	5,266,688	—	—	50
Facilities Construction	16	147	—	152	—	80	1,929,000	—	—	—
2. Shaft Sinking Subcontractor										
Collaring & Headframe	15	255	—	295	—	75	1,050,000	—	—	—
Shaft Sinking & Testing	31,32	517	—	740	431	158	4,410,000	89,040	—	220
Station Construction	26	452	109	611	465	130	1,820,000	187,620	—	312
Raisebore ES-2	3	84	13	100	21	15	420,000	17,010	—	—
3. Test Construction & Support										
Excavation	52	884	884	1,357	1,628	260	7,280,000	1,084,292	—	—
Construction	12	154	84	288	208	60	420,000	63,000	—	—
Test Support	110	616	308	1,232	858	220	3,850,000	77,000	—	—

Most of the items on the inventory list are biodegradable and, therefore, as with the drilling fluid, will support microbial growth. We anticipate that large amounts of water will be used for dust control. Although a list has not yet been prepared, we can assume that many of the same organic materials will be used in the construction of the candidate repository.

The introduction of these organic nutrients is an ongoing process, concomitant to man's activities at the repository, and will not cease until the repository is sealed. The movement of organic contaminants in subsurface environments has been the subject of several excellent reviews.[3-5] Generally, though, the factors governing the transport of organic materials in soils are advection, dispersion, sorption, retardation, and the partitioning of organic contaminants in the aqueous phase.[6]

We directed initial efforts towards confirming that those specific drilling fluids used at Yucca Mountain are susceptible to microbial attack. Once biodegradation was confirmed, research focused on identifying the bacteria isolated from the Nevada Test Site (NTS). Subsequently, we found these bacteria to biodegrade drilling fluids. Finally, studies were performed to determine the growth rates of the microorganisms, changes in pH, oxygen use, and changes in the viscosity of the drilling fluids. We used this information to make concluding statements regarding the effect of microorganisms on ground water chemistry.

II. BIODEGRADATION STUDIES

A. Sources of the Microorganisms

There are several factors that influence the extent and the duration of microbial activity at Yucca Mountain. They are as follows:

1) Water. There is enough water (65% saturation) in the unsaturated zone to support microbial growth.

2) Mineral Content. The mineral content of the water is capable of supporting a microbiological population.

3) Organic Matter. There is very little organic matter present, suggesting that little, if any, organic nutrients exist.

Heterotrophic microorganisms probably exist in the unsaturated zone at a low level of existence (low numbers and metabolic rates). In all probability autotrophic microorganisms exist in the subsurface environment of Yucca Mountain, using reduced inorganic compounds such as FeS_2, H_2 and S^0 as sources of energy. The limiting factor is the low nutrient, or energy source, level. With the addition of nutrients, microbial activity will increase. The duration of higher activity will depend on the concentration of energy source transport of organics along the flow path, the presence of inhibitors, flow rate, and the build-up of toxic by-products.

Microbial activity is simply a function of nutrients and will occur only when nutrients exist. Because of metabolic products, the effects of microbial activity will be a very long term factor. For example, humic acids are produced by microorganisms, are recalcitrant, and are known to combine with metals.[7]

To isolate microorganisms capable of biodegrading drilling fluids, soil samples were collected from two drilling locations at Yucca Mountain (USW H-3 and UE-25c#2). Because these soils had received discharges of drilling fluids during the course of the drilling operations, we felt these locations were appropriate choices. Soil samples were placed in sterile 500-ml, wide-mouth plastic bottles and were immediately returned to the laboratory for analysis. The following mineral salts medium was used to culture these microorganisms:

Salt	g or ml/L	Stock (g/200 ml H_2O)
$NaNO_3$	1.5 g	-
K_2HPO_4	5 ml	1.5
KH_2PO_4	5 ml	3.0
$MgSO_4 \cdot 7H_2O$	5 ml	1.5
Na_2CO_3	5 ml	0.8
$CaCl_2$	10 ml	0.5
$Na_2SiO_3 \cdot H_2O$	10 ml	1.16
Trace elements:		
H_3BO_3	10 µg	-
$CuSO_4$	50 µg	-
$FeCl_3 \cdot 6H_2O$	98 mg	-
$MnCl_2 \cdot 4H_2O$	41 mg	-
$ZnCl_2$	5 mg	-
$CoCl_2 \cdot 6H_2O$	2 mg	-
$NaMoO_4$	4 mg	-
Agar	15 g	-
pH = 7.2	-	-
H_2O	1000 ml	-

To 1.0 l of this medium, either 5 ml of Turco 5622 or 5 ml of ASP-700 drilling fluids was added. Because the drilling fluids were the only energy source, growth on this medium could be interpreted as biodegradation of these fluids. Solid media were inoculated with washings of the soil samples and, following two weeks of aerobic incubation at room temperature, several bacterial colonies were growing vigorously on the medium. To obtain pure cultures, isolated colonies were transferred to fresh media and incubated for an additional two weeks. Colony morphology and microscopic characteristics were then determined, and this information is presented in Table IV.

TABLE IV

CHARACTERIZATION OF COLONY AND MICROSCOPIC MORPHOLOGY

Culture No.[a]	Colony Morphology	Microscopic Morphology
1	Tiny isolated colonies, mixed culture.	G- long, thin rods.[b] Blue ends.
2	Large white colonies. Lens-like appearance.	G- rod. Short, almost cocci. Sometimes pairs.
3	Similar to No. 2. Could be contaminated with No. 1.	Same as No. 2.
4	Small, isolated colonies, similar to No. 1. Pure.	G- long, thin rods. No blue ends.
5	Same as No. 3.	G- rods of varying lengths. Similar to No. 1.
6	No growth.	—
7	Similar to No. 2, but not as white.	G- cocco-bacilli with contaminants.
8	Similar to No. 1, not contaminated.	G- rods, medium long and thin. Large groups.

Culture No.[a]	Colony Morphology	Microscopic Morphology
9	Many isolated small colonies, no color.	G- small, thin, rods. Contaminated with G+ cocci.
10	Odd growth. Colony in groove of inoc. needle. Mucoid. White.	G- tiny, thin rods. In large groups, randomly.
11	Mucoid growth, only at initial inoculation. White.	G- rods and cocci. Heavy encapsulation.
12	Same as No. 11, but not as much growth.	Mixture of G- rods and G+ cocci.
13	Same as No. 12.	G- fat rods. Encapsulated.
14	Heavy growth. Swarm-like, spreading out from initial inoc.	G- rods. 1) short, thin; 2) shorter, fat (with blue ends).
15	Similar to No. 9.	G- rods with G+ cocci/rods,
16	Heavy growth. Mucoid white colonies.	Similar to No. 15.

[a] Numbers 1-8 are cultures growing on Turco 5622, 9-16 on ASP 700.
[b] G- is gram negative, G+ is gram positive

Clearly, several species of microorganisms indigenous to the NTS are capable of biodegrading drilling fluids. In subsequent studies, it had been determined that many of these species are the same or that some species were really two or more species. Since then we have shortened the list to 13 species of bacteria, 9 of which biodegrade ASP-700 and 4 that biodegrade Turco 5622 fluids. From these species, seven were sent to Dr. Myron Sasser's laboratory at the University of Delaware, Newark, Delaware, for further identification. Briefly, the results are as follows:

Our Code	Most Likely Match	Similarity
11_c	Pseudomonas stutzeri	0.461
9_{A8b}	Pseudomonas mendocina	0.154
9_{A2b}	Pseudomonas mendocina	0.253
9_{A5}	Pseudomonas mendocina	0.056
9_{A5}	Pseudomonas mendocina	0.047
1_A	Agrobacterium tumefaciens	0.049
S.P.2	Alteromonas putrefaciens	0.281
11_c	Pseudomonas mendocina	0.302

As can be seen, the similarity among the species that we submitted and the reference species is quite low (in which 1.0 is perfect correlation and 0.0 is no correlation). In addition, we submitted organisms 11_c under two different names and received different results. We tend to believe that this discrepancy is due to the difficulty in bacterial taxonomy associated with the genus Pseudomonas, and also believe Dr. Sasser's staff did an admirable job in attempting to identify these bacteria. Because most of our studies have dealt with the 11_c bacterium, we have done extensive taxonomic analyses of this species. Table V expresses the results of our investigation.

TABLE V

COMPARISON OF ORGANISMS 11_c TO OTHER *PSEUDOMONAS* SPECIES

Tests	11_c	*P. stutzeri*	*P. alcaligenes*	*P. Inendocina*	*P. aeroginosa*
Morphology (rods)	+	+	+	+	+
Motility (very motile)	+	+	+	+	+
Gram-stain	-	-	-	-	-
Growth @ 4°C	-	-	-	-	-
Growth @ 43°C	+	+	41°C	41°C	41°C
Poly-β-hydroxy butyrate	-	-	-	-	-
Egg yolk reaction	-	-	-	-	-
Fluorescent pigment	-	-	-	-	-
Starach hydrolysis	+	+	-	-	-
Arginine dehydrolase	+ (weak)	-	+	+	+
Gelatin hydrolysis	+	-	+	-	+
Denitrification	+	+	-	+	+
Carbon sources					
Dextrose	+	+	-	+	-
Geraniol	-	-	-	+	+
L-valine	-	+	-	+	-
Maltose	+	+	-	-	-
D-mannitol	+	d	-	-	+
Acetate	+	+	+	+	+
D-L malate	+	d	d	d	d
Ethylene glycol	+	+	-	+	-
Butyrate	+	+	d	+	+

In addition to the above analyses, 11_c was found to grow on the following substrates: propionate, stearate, maleiate, citrate, mannitol, ethanol, pyruvate, casein, casamino acids, glycine, aspartate, yeast extract, choline, betaine, Tween 80, Span 80, and Tween 61. Ethylene glycol (Table V), Tween 80, Tween 61, and Span 80 are components of the drilling fluids used at Yucca Mountain. No growth was observed on pthatate, sorbitol, tartrate, EDTA, oxalate, sucrose, benzoate, nicotinate, cellulose, polyethylene glycol (3550), acrylamide, methanol, and pantothenate. Also, 11_c is a strict aerobe; and in the complete absence of oxygen it does not ferment glucose, nor does it grow with acetate as the energy source and nitrate, nitrite, iron (III), sulfate, or sulfur as electron acceptors.

Our examinations lead us to believe that microorganism 11_c is a strain of *Pseudomonas stutzeri*. The other microorganisms are closely related to those species suggested by Dr. Sasser. The identification of those microorganisms found to biodegrade the drilling fluids was important because that information will aid in understanding the types of sorption being performed by the microorganisms and in understanding the potential for metal mobilization.

B. Growth Studies

It was not only important to determine which microorganisms are able to use drilling fluids as growth substrates, but also it was equally important to determine the rate at which microorganisms grow. Growth studies can provide useful information regarding the potential of the drilling fluids to support a

population of microorganisms, and the duration of microbial activity. Two methods were used to determine growth rates: optical density and oxygen uptake measurements. Optical density measurements were performed on those species growing in Turco 5622 medium (the Nalco ASP-700 medium is too opaque to transmit light), and oxygen uptake measurements were performed on both drilling fluid media. For the optical density measurements, 5.0 ml of a 24-h culture was added to 25 ml of sterile medium in a 250-ml side arm-flask. Readings were taken every 24 h for 144 h (6 days) by using a Klett-Summerson photometer. Data are presented in Klett Units (Fig. 1), for which 10 Klett Units represent approximately 1×10^7 bacteria per milliliter and 30 Klett Units represent approximately 1×10^8 bacteria per milliliter. All optical density studies were performed at room temperature (18-20°C).

In respiration studies, five drops of a 24-h culture were added to 2.5 ml of broth in a Warburg manometer. Oxygen use is determined by measuring the decrease in head space gas volume over time. Figures 2 and 3 show the results as micromoles of oxygen consumed per hour per milliliter of medium. All oxygen consumption studies were performed in a water bath at 20°C.

Both the optical density and oxygen use results demonstrated distinct growth rates. For the optical density measurement, the bacteria grew rapidly for 48 h and then growth slowly increased for the duration of the experiment. Oxygen consumption rates for the same species showed a similar pattern. The greatest use of oxygen occurred during the first 6 h of growth. It should be noted that oxygen consumption does not correlate directly with optical density because the greatest use of oxygen occurs when the optical density is low, at the early stages of growth where the young cultures are consuming O_2 at a much higher rate than older cultures. Although there was a temperature difference between the two experiments, it probably was not enough to make a real difference in the results.

The following data were obtained from earlier experiments done with mixed cultures:

Hours	$\mu mol \cdot h^{-1} \cdot g^{-1}$
24	893
48	663
72	603
96	451
120	404
144	222
168	75
192	15

With time, the rate of oxygen use decreased. During the entire experiment, 8.14×10^4 μM of oxygen was consumed per gram of drilling fluid (per 192 h). Because one gallon of drilling fluid (diluted 1/42 with water, the dilution used by the drillers at Yucca Mountain) contains 90 g of polymer, we anticipate that 7.31 M O_2 would be consumed for every gallon of polymer, under optimal conditions (8.12×10^4 μM O_2/g x 90 g/gal = 730.8×10^4 μM O_2/gal, or 7.31 M O_2/gal). Considering that millions of gallons of drilling fluids have been used during the site characterization process, there is a strong potential for oxygen depletion and a concomitant drop in the oxidation-reduction potential.

C. Viscosity

A long-term experiment was performed investigating viscosity changes of ASP-700 and Turco 5622 medium. Five bacterial species were grown individually

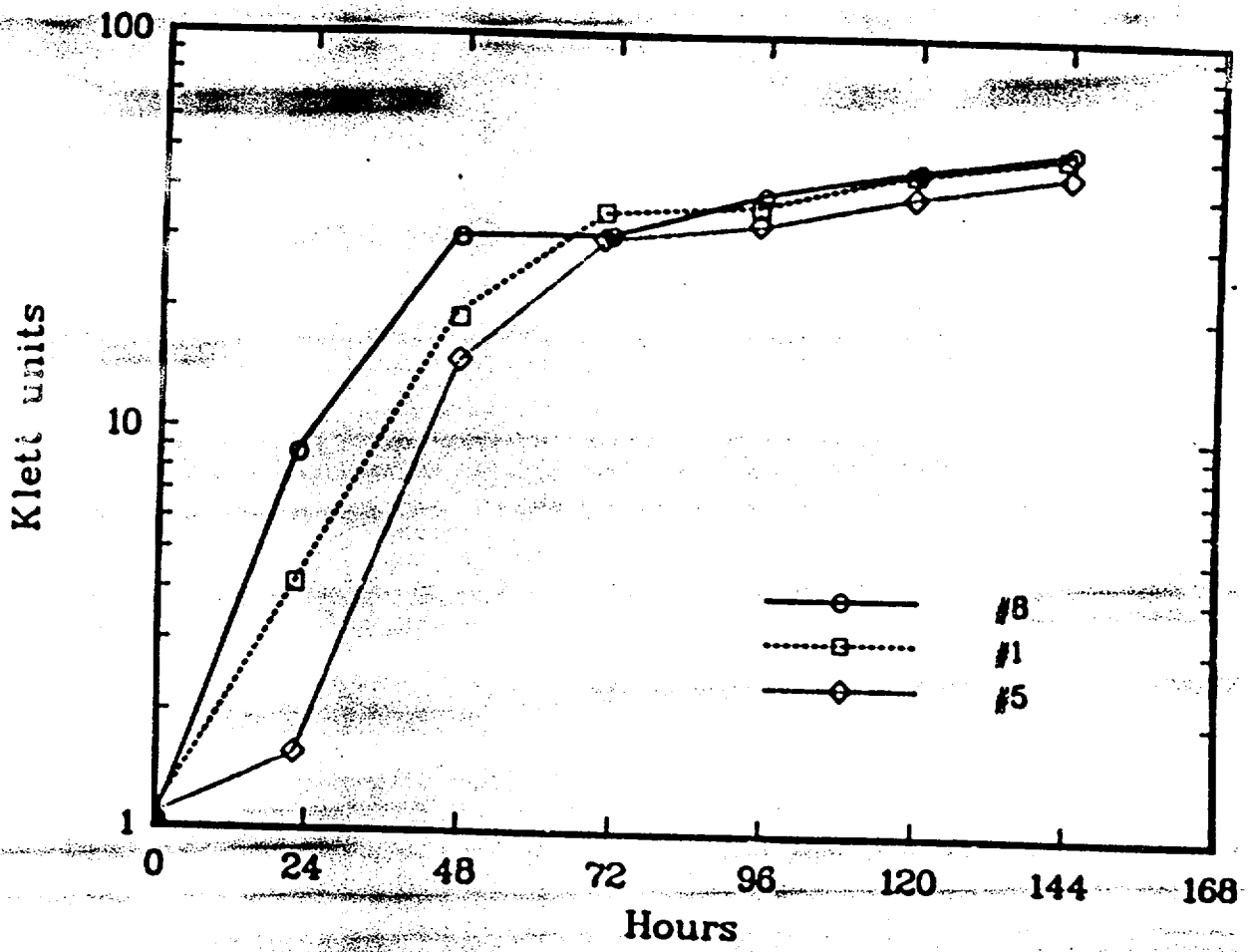

Fig. 1. Growth of three bacterial isolates in Turco 5622, measured by a Klett-Summerson photometer.

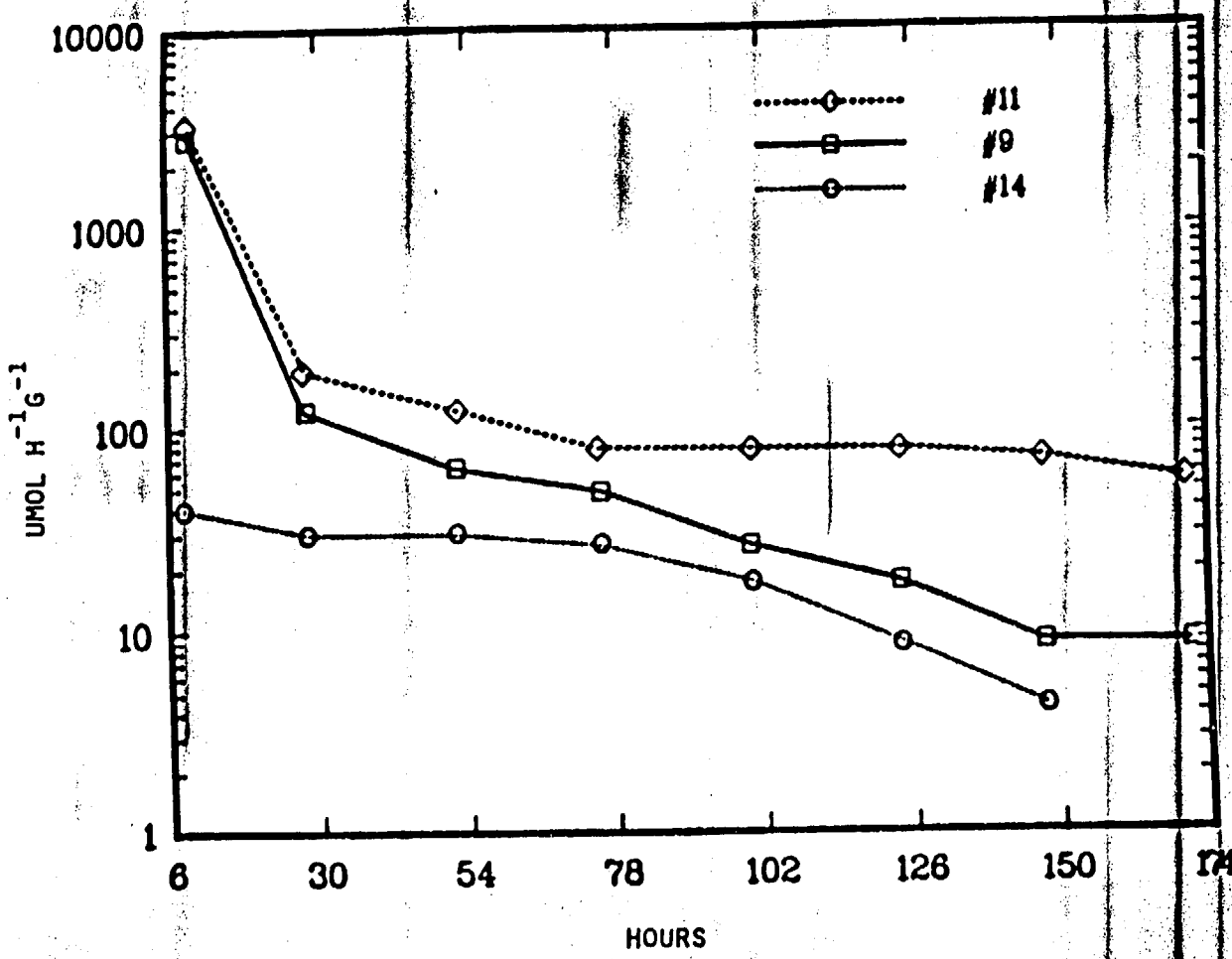

Fig. 2. Oxygen consumption of three bacterial isolates in ASP-700, measured by Warburg manometers.

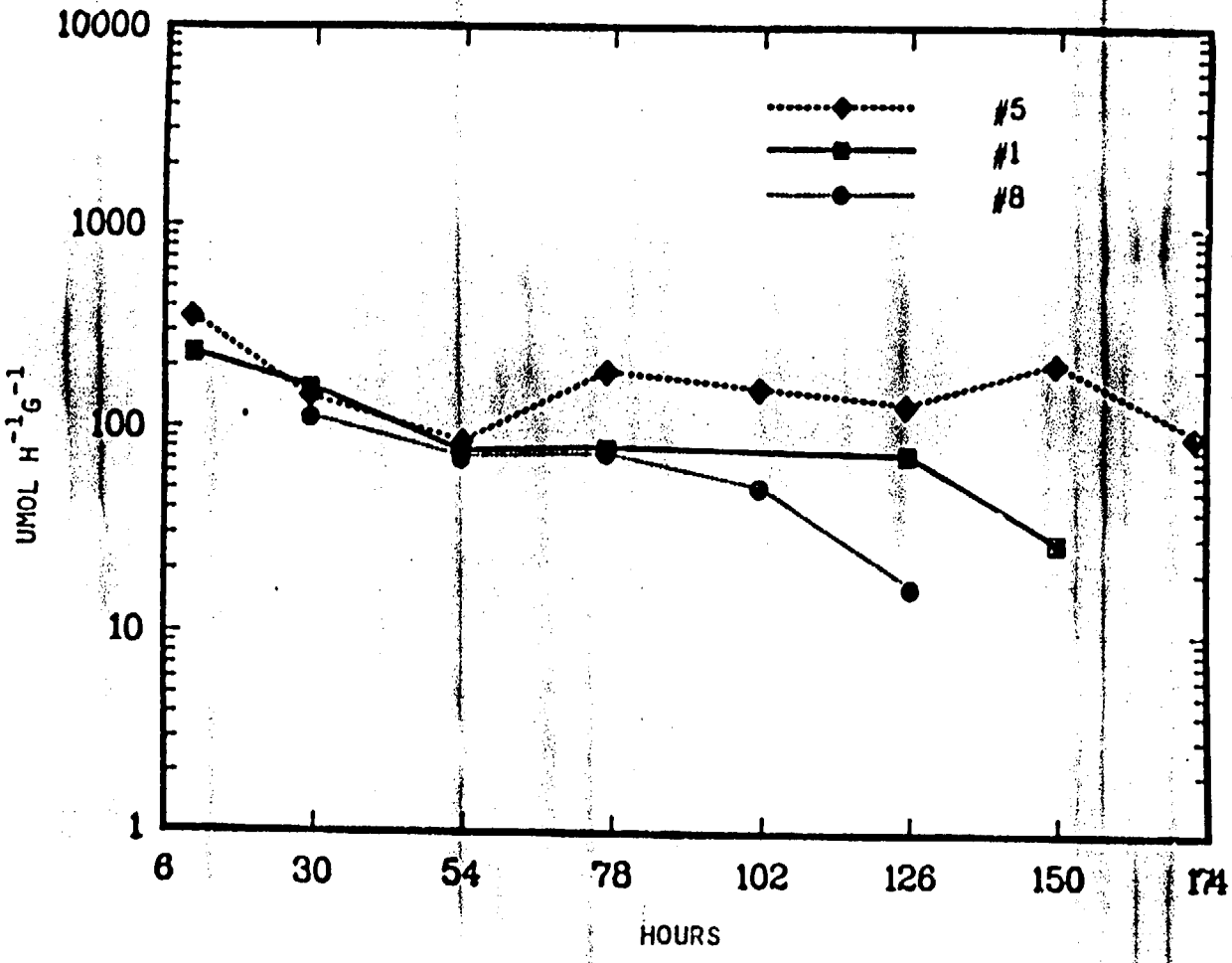

Fig. 3. Oxygen consumption of three bacterial isolates, in Turco 5622 measured by Warburg manometers.

in the ASP-700 medium, while two species were grown in the Turco 5622 medium. For each species, 180 ml of medium was inoculated with 18 ml of a 48-h culture. These flasks were mixed at 60 rpm for 25 days at 22°C. Twenty-five milliliters of media was withdrawn on days 0, 3, 7, 12, 18, and 25 for viscosity measurements. To measure the viscosity of the ASP-700 medium, a small glass bead (2.9-mm diameter, 0.030399 g) was dropped through a glass tube (11.4-mm inside diameter by 20 cm) containing the medium. The fall of the bead through the 20-cm distance was timed, and any change in travel time was assumed to be indicative of a change in viscosity, relative to non-inoculated control. For the Turco 5622 medium, the rise of air bubbles through the medium was used to measure viscosity. Air bubbles were injected into the medium using a 10-μl, precision syringe and, as with the ASP-700 medium, the travel distance was 20 cm. The results of this experiment are presented in Table VI. Any increase in the values reported over time can be interpreted as a relative increase in the viscosity of the medium. Conversely, as the numbers decrease with time, we could assume that the viscosity of the medium was decreasing.

TABLE VI

THE EFFECTS OF BACTERIAL GROWTH ON THE VISCOSITY OF ASP-700 AND TURCO 5622 MEDIUM[a]

Day	ASP-700					Turco 5622	
	$^{9}A_{2b}$	$^{9}A_{3}$	$^{9}A_{5}$	$^{11}A_{2a}$	$^{11}A_{2b}$	^{1}A	^{6}A
0	0.93	0.93	0.91	0.92	0.90	1.32	1.24
3	0.94	0.99	0.96	0.94	0.95	1.01	0.98
7	0.91	0.98	0.92	0.91	0.92	—	1.25
12	0.92	0.99	0.95	0.90	0.90	1.33	1.19
18	0.72	0.75	0.74	0.71	0.70	1.51	1.75
25	0.80	0.84	0.86	0.75	0.75	1.33	1.21

[a]Results are expressed as a ratio of the unknown value divided by the control value.

Apparently, as the bacteria grew in the ASP-700 medium, the viscosity decreased. This was true for all five of the species that were tested. Following an initial decrease, the viscosity of the Turco 5622 medium tended to increase with time until day 25 for which the values were similar to day 0. These results suggest that more than one constituent of the Turco 5622 medium was being acted upon by the microorganisms, which in turn had a varying effect on viscosity.

D. pH

The pH changes in the drilling fluid medium were recorded for 10 species of bacteria. The pH was recorded after 10 days of growth in either ASP-700 or 5622 media. The results are as follows:

Species	pH	Assigned Species
ASP-700 control	7.98	
9_{A5}	7.98	P. mendocina
$9_{A2b}C$	7.72	

Species	pH	Assigned Species
11_A	8.06	
11_C	8.03	P. stutzeri
9_{A2b}	8.30	P. mendocina
9_{A2b} N.C.M.C.	8.23	P. mendocina
Turco 5622 control	7.68	
Stag pond "B" org 2	7.73	
6_A	7.69	
1_A	7.59	A. tumefaciens
Stag pond #2	7.66	A. putrefaciens .

With few exceptions, bacterial growth had little effect on pH. Species 9_{A2b} and 9_{A2b} both increased the pH of ASP-700 medium by 0.2 and 0.3 pH units, respectively.

E. Survival.

A simple experiment was performed to determine the longevity of microorganism 11_C. One milliliter of a 24-h culture was added to 50 ml of nutrient broth and maintained at room temperature. Optical density and colony-forming unit measurements were recorded periodically over several months. The initial culture was inoculated on October 10, 1985, and as of December 30, 1985, the study was still being conducted. We have estimated that this microorganism will survive in nutrient broth for over 2 years.

Using a light microscope, we observed that after 18 h of incubation, the cell morphology of this culture changed from a rod to small rod or cocci. This shrinking of bacteria was consistent with the finding of Humphrey et al.[8] who have reported "dwarfism" in starving marine bacteria. The bacteria apparently enter this stage as a means of coping with a harsh environment, and there may be a relationship between the decrease in size and the survival of the bacteria.

These findings indicate that this bacterium, a Pseudomonas sp., is well suited for long-term survival at the Nevada Test Site.

III. SORPTION STUDIES

Microorganisms are known to accumulate a variety of metals as a means of protection against the toxic effects of the metals. The simplest protection mechanism used is the precipitation of the metal on the outer surface of the microorganism. This precipitation occurs because of the activities of membrane-associated sulfate reductases,[9] or through the biosynthesis of oxidizing agents such as oxygen or hydrogen peroxide.[10] In this way, metals such as iron are deposited on the surfaces of many species of bacteria, uranium on the surface of fungi and iron, nickel, copper, aluminum, and chromium on the surface of algae. A second means of surface accumulation is through the production of extracellular ligands that complex metals outside the cell and prevent their uptake. Both cyanobacteria and green algae have been found to concentrate nickel 3000 times more than the original metal in the culture medium. The binding of metals proves to be rather specific, with charge, ionic radius, and coordination geometry the predominant factors. Another measure adopted by microorganisms to prevent metals from reaching toxic levels is the biosynthesis of intracellular traps for the removal of metal ions from solution; for example, the biosynthesis of the sulfhydryl-containing metallothionein protein to bind cadmium and copper.[11]

By depositing metals, internally or externally, microorganisms are not only protecting themselves from the toxic effects of the metal ions, but are also, in effect, concentrating the metal in the biosphere. Because of the strong evidence in the literature, it is our concern that microorganisms are also capable of sorbing radioactive wastes.[12-25] Strandberg et al. have described the intracellular accumulation of uranium by the bacterium Pseudomonas aruginosa.[26] Interestingly, the uptake of uranium is quite rapid, as if mediated by an active transport system even though a uranium transport system has not yet been described. In the case of plutonium, Neilands and Raymond et al. have suggested that it could be moved into the cell via the well-described siderophore iron transport mechanism.[26,27] The siderophore transport system could be moving Pu^{4+} in an analogous manner to Fe^{3+}, because of a similarity in their ionic radius/charge ratio.

Because of the evidence presented above, we investigated the effects of microorganisms on the uptake of radioactive wastes, particularly from the standpoint of enhanced metal mobilization.

A) Media. Bacteria were grown and maintained on a minimal salts medium that contained 1.0% of the ASP-700 (w/v) drilling fluid, which is used as an energy and carbon source.

B) $^{239}Pu^{4+}$ Feed Solution. Twenty-five milliliters of 0.1 m $^{239}Pu^{4+}$ solution tracer developed at Los Alamos National Laboratory was transferred into a washed Pyrex centrifuge cone containing 1 ml of saturated $NaNO_2$ solution. The test tube was then placed in a heater block until the liquid had evaporated completely. The $^{239}Pu^{4+}$ residue was resuspended in an appropriate amount of 0.1 N HCl and transferred to a washed 50-ml Oak Ridge-type, polycarbonate centrifuge tube to air dry. Filtered (0.05 μm) J-13 well water was used to resuspend the $^{239}Pu^{4+}$ to a final concentration of 10^{-3} to 10^{-9} M ^{239}Pu. The oxidation state of the ^{239}Pu was predominantly 4+, although some 5+ and 6+ may have also been present.

C) Sorption. The Pseudomonas sp. was transferred from a streak plate (nutrient agar, Difco Co.) to a 250-ml Belco culture flask containing 50 ml of nutrient broth (Difco). The flasks were incubated at room temperature for 18 h. One milliliter of broth was transferred to another 250-ml flask containing 49 ml of nutrient broth. Again, the culture was incubated at room temperature for 18 h then used to inoculate fifteen 250-ml Belco side-arm flasks, each containing 49 ml of nutrient broth. The flasks were incubated at room temperature for periods of 3, 6, 12, 24, or 48 h. After each incubation period, we removed three flasks and determined the optical density of each culture by using a Klett-Summerson photometer, which was equipped with a #54 filter (520- to 580-nm transmission). We then determined the number of viable cell counts (or colony-forming units) using standard-dilution and pour-plate methods. The total broth volume from each side-arm flask was transferred to a 50-ml polycarbonate Oak Ridge-type centrifuge tube and centrifuged at 17,300 g for 10 min. The supernatant was discarded and the bacterial pellet was washed with 10 ml of phosphate buffer. The tubes were again centrifuged at 17,300 g for 10 min. This process of washing the pellet was performed a total of three times. After the final washing, the supernatant was discarded and 20 ml of $^{239}Pu^{4+}$ feed solution was added to each of the three tubes. The tubes were agitated at 100 rpm at room temperature for 5 days. The tubes were centrifuged at 17,300 g for 1 h, then the supernatant was transferred to the Oak Ridge-type 50-ml centrifuge tube and centrifuged again at 17,300 g for 1 h. The pellets were air dried at room temperature and weighed. Following the 1-h centrifugation, 10 ml of supernatant was transferred to another Oak Ridge-type centrifuge tube and centrifuged at 17,300 g for 2 h. Five milliliters of supernatant was

removed and transferred to a 20-ml scintillation vial to which 5 ml of water and 10 ml of Insta-Gel liquid scintillation cocktail (United Technologies, Packard Company) were also added. The vials were mixed and placed in a Seale Mark II scintillation counter for 50 min. The window opening was set at 1 to 99 minimum, the maximum gain was set at 000; the range switch was set at F; and the scaler was turned to the off position.

D) Sorption Ratio. The activity of each vial was determined three times. These values were averaged and the sorption ratio (activity in solid phase/activity liquid phase) was determined as follows: Background count was subtracted from the experimental count, yielding the activity in the liquid phase. We then subtracted the liquid phase activity count from the feed solution activity count, yielding the activity in the solid phase. We multiplied the solid phase activity count by 4 (because only 5 of the initial 20 ml of feed solution was counted) and divided by the product of the dry weight of the bacteria times the activity in the liquid phase. The written equation reads as follows:

$$\text{Sorption Ratio} = \frac{4 \times (\text{activity in the solid phase})}{(\text{dry weight}) \times (\text{activity in the liquid phase})} .$$

We performed three replicates for each experiment, with each experiment repeated three times. Although our data are presented as sorption ratios, there exists no information to demonstrate that sorption is actually occurring. What we observed is the bacteria removing the plutonium from solution. These experiments serve to demonstrate that these bacteria are able to remove $^{239}Pu^{4+}$ from solution, and are not definitive regarding the mechanism or rate of removal.

E) Bacterial Growth. Table VII presents the data for the optical densities, dry weights, and colony-forming units of the bacteria. Each set of measurements demonstrates that the Pseudomonas sp. grew rapidly, reaching 2.1×10^9 cells ml^{-1} (or cells/ml) in 12 h of incubation. The cell numbers slowly increased to 3.4×10^9 cells ml^{-1} in 48 h. Clearly, this species was able to maintain a high cell concentration over a prolonged period of time. Interestingly, when these cells were examined under a light microscope, we observed over time a change in their morphology. At 3, 6, and 12 h, the cells were rod shaped; however, after 18 h of incubation, the cells decreased in size and became coccoid in shape.

F) Sorption of $^{239}Pu^{4+}$ by the Pseudomonas sp. Table VII expresses the results of the sorption experiments. Perhaps the most outstanding feature of the data is the strong sorption of the $^{239}Pu^{4+}$ by Pseudomonas sp. The bacteria concentrated the $^{239}Pu^{4+}$ nearly 1000 times greater than did the sterile control (1 g of sterile, crushed tuff, 75-200 mesh). Sorption also appears to be related to the physiological state of the bacteria. On a per-gram, dry-weight basis, the 3-h culture sorbed the most $^{239}Pu^{4+}$, followed closely by the 6-h cultures. We observed the lowest sorption ratio for the 12-h and 24-h cultures, followed by a slight increase in sorption by the 48-h cultures.

TABLE VII

OPTICAL DENSITY, DRY WEIGHTS, COLONY-FORMING UNITS AND SORPTION RATIO FOR A PSEUDOMONAS SP. DURING 48 h OF INCUBATION

Incubation Time (h)	Optical Density (Klett Units)	Dry Weight (g)	Colony-Forming Units CFUs (per ml)	Sorption Ratio (ml/g ± sd)
3	7.53	0.00080	1.0×10^7	9,280 ± 707
6	26.8	0.00179	8.8×10^7	9,840 ± 4,417
12	174	0.01411	2.1×10^9	2,156 ± 161
24	173	0.01474	2.7×10^9	2,080 ± 230
48	155	0.01547	3.4×10^9	4,900 ± 342
Control (1 g crushed tuff)	—	1.000	—	5.37 ± 0.56

IV. DISCUSSION OF BIODEGRADATION AND SORPTION

The major results of our biodegradation investigations show that the millions of gallons of drilling fluids used at Yucca Mountain are biodegradable; that these drilling fluids will support a large population of microorganisms that use measureable amounts of oxygen; and that these bacteria are capable of surviving for long periods of time. The presence of microorganisms can have profound effects on the quality of groundwater chemistry. Both inorganic and organic chemicals entering the groundwater environment can be transformed by microbiological processes. To obtain energy for growth, microorganisms can oxidize organic compounds, or reduced forms of hydrogen iron, nitrogen, or sulfur. Electrons from these oxidation reactions are transferred to electron acceptors, such as oxygen under aerobic conditions and nitrate, sulfate, and carbon dioxide under anaerobic conditions. In addition to transforming chemical compounds, microbial growth can change the permeability of the porous aquifer material and affect the chemical environment by changing the pH and oxidation-reduction potential of the system. Such changes can lead to other strictly chemical phenomena, such as precipitation or dissolution of phosphates and heavy metals or chemical oxidation or reduction of iron and sulfur salts. Thus, many physical and chemical changes can be brought about in association with the decomposition or transformations of organic and inorganic materials by microorganisms living in the subsurface environment.

The intent of our research was not to investigate the direct effects of microbiological growth on every aspect of groundwater chemistry; such an investigation was beyond the capabilities of the NNWSI Project. The effects of microbial activity on groundwater chemistry are well documented in the

literature of Britton and Rheinheimer.[29-30] Rather, our investigation demonstrated there is potential for significant and long-term microbial activity in the groundwater at Yucca Mountain. The investigation also emphasized that strong scientific evidence indicates that such activity often has a profound effect on groundwater chemistry. Based on the information gained from the literature and our laboratory studies, the following approximations can be made regarding water chemistry:

1) pH. There exists no evidence that microbial activity will change the pH of the groundwater. All of our studies regarding pH resulted in little or no change in the pH of the growth media. In addition, the concentration of bicarbonate in the groundwater serves as a strong buffering agent. Therefore, pH should be unaffected by microbial activity.

2) Oxidation-Reduction Potential. There is a strong possibility that microbiological activity could result in reducing conditions, at least in localized environments. Our experiments have consistently yielded information confirming oxygen uptake. However, we have little information regarding the long-term stability of reducing conditions.

3) Viscosity. Depending upon the drilling fluid, viscosity is either increased or decreased. Because viscosity, as affected by microbial activity, is also dependent upon the species of bacteria, it would be very difficult to predict the overall effect of microbial activity on viscosity at Yucca Mountain.

Thus, the most important effect that microorganisms may have on the groundwater chemistry of Yucca Mountain is the removal of oxygen and the concomitant chemistry in oxidation reduction potential.

During our sorption studies, it is interesting that we never observed the classical log death phase of organism growth. Normally, researchers would anticipate that after 24 h, because of nutrient depletion and the build-up of toxic by-products, cell viability would begin to rapidly decrease. The bacteria, however, increased in numbers throughout the duration of the experiment.

The observed shrinking of the bacteria is consistent with the finding of Humphrey et al., who have reported "dwarfism" in starving marine bacteria.[8] The bacteria apparently enter this stage as a means of coping with a harsh environment, and there may be a relationship between the decrease in size and the survival of the bacteria.

The possibility that the bacteria may sorb the $^{239}Pu^{4+}$ is not surprising because, as previously mentioned, bacteria are known to sorb many different metals from solution. These results are consistent with the findings of Strandberg et al. who reported a strong intercellular uptake of uranium by Pseudomonas aeruginosa.[26] The most interesting finding of our study is that sorption appears to be dependent upon the physiological state of the bacteria. The results show that the younger cultures sorb the $^{239}Pu^{4+}$ more strongly than the older cultures. At 3 h, these cultures were entering log growth phase, during which cells rapidly grow and divide. At 6 h, the cultures entered log growth phase and, here again, the $^{239}Pu^{4+}$ sorption was quite high; however, by 12 h, $^{239}Pu^{4+}$ sorption declined rapidly. These events appear to coincide with the onset of stationary phase, during which growth and division no longer occur and death of the cells has not yet begun. At 24 h, the sorption rate was also low and, as indicated by the cell number and optical density measurements, the cultures were in stationary phase. At 48 h, the cultures were still in stationary phase but for some poorly understood reason sorption began to increase. This observation is supported by the results of preliminary experiments, during which sorption was quite strong at 54 h of incubation. One

possible explanation could be that after prolonged growth, the organisms have entered severe nutrient depletion and are devoting much of their energy to mineral uptake, resulting in the $^{239}Pu^{4+}$ being taken up in a manner analogous to the essential Fe^{3+} cation. The significant sorption coefficients obtained by the 3- and 6-h cultures may also be due to $^{239}Pu^{4+}$ being taken up similarly to a needed mineral. Alternatively, the $^{239}Pu^{4+}$ may be toxic to the cells and the cells are more capable of internally sequestering the plutonium at 3 and 6 h than they are at 12 and 24 h. Finally, the sorption could also be the result of a surface precipitation mediated by an electromagnetic effect or accumulation by a protein. With age, the surface characteristics of a bacteria change, and this change could strongly affect $^{239}Pu^{4+}$ sorption.

Regardless of the means by which $^{239}Pu^{4+}$ is sorbed by the bacteria, the fact remains that these bacteria are able to remove this actinide from solution. What remains to be determined is the overall effect that this biological sorption may have on the movement of radioactive wastes from a high-level nuclear waste repository. Studies show that bacteria will be removed from suspension by soil or rock. In most classical saturated flow conditions, this is probably true; however, it is unclear what effect the surrounding rock will have on the bacteria if saturated or unsaturated fracture flow conditions are predominant. Most bacteria are motile and, therefore, are not confined to an isolated location. We must emphasize that there exists little information regarding the extent of microbial activity in the deep, subsurface environment (B. Ghiorse, Cornell University, personal communication). It has always been assumed that microbial activity occurred only in the first few meters below the soil's surface. However, recent advances in sampling techniques have kindled new interest in the subject, and it is now known that significant microbial activity can occur to depths of 30 m or more below the surface. Unfortunately, these sampling techniques cannot access microbial activity at greater depths; therefore, the extent of microbial activity at the depths of the candidate high-level nuclear waste repository is still subject to considerable conjecture.

What we have learned so far in our studies of microbial effects on radioactive wastes is considerable. We know that several species of bacteria are capable of aerobic biodegradation of the drilling fluids used at the Nevada Test Site, and we know that at least one of the species is capable of strongly sorbing $^{239}Pu^{4+}$. What researchers need to investigate is the fate and mobility of the sorbing bacteria in the environment and the extent of radioactive waste sorption among other members of the microbiological community. Once these two areas are more clearly understood, we can begin to make meaningful predictions regarding the contribution of microorganisms to the retardation of radioactive wastes in tuff.

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BIODEGRADATION OF DRILLING FLUIDS: EFFECTS ON ACTINIDE SORPTION

Larry E. Hersman, LS-3

Abstract

A Pseudomonas sp., isolated from the Nevada Test Site, was used to study the removal of ^{239}Pu from solution. Bacteria were grown in nutrient broth, harvested and mixed with 10^{-8} M ^{239}Pu feed solution. The results clearly demonstrate that bacteria sorb the actinide. In addition, sorption appears to be dependent upon the physiological state of the bacteria, with the younger cultures (3 and 6 h) sorbing at a higher rate than the older cultures.

Introduction

During the process of characterization, by the Nevada Nuclear Waste Storage Investigations (NNWSI) Project, of the geology and hydrology of the site for a candidate high-level nuclear waste repository at Yucca Mountain, Nevada, several million gallons of drilling fluids were used. Within a 3-km radius of the exploratory shaft it is estimated that nearly 15,000,000 gallons of drilling fluids were released into the block (candidate site), or in the immediate area.[1] The drilling logs from these wells indicate that a significant amount of the fluid was lost in the Topopah Spring Member, which would be the geologic location of the repository. The Topopah Spring Member

has been characterized as a welded tuff with a low permeability and a high content of fractures. Montazer and Wilson[2] have therefore suggested that in this member, lateral movement may exceed downward rates, a statement that was substantiated by the recent discovery of drilling fluids in the USW UZ-1 hole (air drilled without fluids). Interestingly UZ-1 is located several hundred meters upgradient from the closest fluid drilled holes (USW G-1 and H-1) and its drilling was limited to the unsaturated zone, at a depth approximately equal to that of the repository.

In addition to those drilling fluids, several other forms of organic materials will be introduced into the block. During the construction of the Exploratory Shaft, items such as diesel fuels and exhausts, hydraulic fluids, and additional drilling fluids will be discarded routinely. It can also be anticipated that much of the same materials will be used during the construction of the repository itself. Therefore, before the repository is completely sealed, there will be a significant amount of organic material located near it.

Our specific concern is that these organic materials may be utilized as growth substrates by a large number of microorganisms, which in turn may influence the transport of radioactive elements away from the repository. Microorganisms can affect transport in one or more of the following ways:

1) alter the composition of the groundwater chemistry through changes in pH or Eh,
2) solubilize radioactive elements by producing chelating agents,
3) transport the radionuclide via biological movement,
4) transport the radionuclide via colloidal dispersion,
5) retard the transport of the radionuclide by sorption onto a nonmotile solid phase.

The microbiological research being performed as part of the NNWSI Project can be broken down into three sequential research tasks. First, it is important to determine if the drilling fluids and other exogenous organic materials are biodegradable. Second, the potential for sorption of radioactive elements by these biodegrading organisms must be determined. Finally, the effect of microorganisms on transport should be documented. The results of the first task have, for the most part, been completed and are present in the milestone report titled "Biodegradation of Drilling Fluids: Effects on Groundwater Chemistry". The second task is now being performed, and the results obtained to date are presented in this paper. Research on the third task is scheduled to begin in the near future.

Microorganisms have been shown to accumulate a variety of metals as a means of protection against the toxic effects of the metals. The simplest mechanism used is the precipitation of the metal on the outer surface of the microorganism. The precipitation of the metal occurs because of the activities of membrane-associated sulfate reductases,[3] or through the biosynthesis of oxidizing agents such as oxygen or hydrogen peroxide.[4] In this way, metals such as iron are deposited on the surfaces of many species of bacteria: uranium on the surface of fungi; and iron, nickel, copper, aluminum, and chromium on the surface of algae. A second means of surface accumulation is through the production of extracellular ligands that complex metals outside the cell and prevent their uptake. Both cyanobacteria and green algae have been found to concentrate nickel to 3000 times the concentration in the culture medium. The binding of metals has been shown to be rather specific, with charge, ionic radius, and coordination geometry being the predominant factors. Another measure adopted by microorganisms to prevent metals from

reaching toxic levels is the biosynthesis of intracellular traps for the removal of metal ions from solution. One example is the biosynthesis of the sulfhydryl-containing metallothionein protein to bind cadmium and copper.[5]

By depositing metals, internally or externally, microorganisms are not only protecting themselves from the toxic effects of the metal ions, but are also, in effect, concentrating the metal in the biosphere. Because of the strong evidence in the literature, it is our concern that microorganisms are also capable of sorbing radioactive wastes as well[6-19]. Strandberg et al.[14] have described the intracellular accumulation of uranium by the bacterium Pseudomona aruginosa. Interestingly, the uptake of uranium is quite rapid, as if mediated by an active transport system even though a uranium transport system has not yet been described. In the case of plutonium, Neilands[20] and Raymond et al.[21] have suggested that it could be moved into the cell via the well described siderophore iron transport mechanism. The siderophore transport system could be moving Pu^{+4} in an analogous manner to Fe^{3+}, due to a similarity in their ionic radius/charge ratio.

Because of the evidence presented above, we felt it necessary to investigate the effects of microorganisms on the uptake of radioactive wastes, particularly from the standpoint of enhanced metal mobilization.

MATERIALS AND METHODS

Bacteria. A bacterium isolated from the Nevada Test Site was found to biodegrade ASP-700 drilling fluid (Nalco Chemical Company). Based on the results of a standard API 20E test strip, the bacterium has been tentatively

identified as Pseudomonas fluorescens. A culture has been sent to the American Type Culture Collection for a more extensive identification. The bacterium has been maintained on ASP-700 medium (described below) at room temperature with transfers performed on a bi-monthly basis. Streak plate analyses were performed periodically to insure that the culture was free from contaminants.

Media. Bacteria were grown and maintained on a minimal salts medium that contained 1.0% of the ASP-700 (w/v) drilling fluid as an energy and carbon source. The constituents of the drilling fluid were as follows (on a percentage, w/v basis): water, 43.2; polymer (acrylamide-sodium acrylate copolymer), 28.9; light hydrocarbons (hexane, pentane, etc.), 25.8; non-ionic emulsifier (50% Tween 61, 50% Span 80), 2.0; and EDTA (ethylenediaminetetra-acetic acid), 0.0135. The constituents of the minimal salts solution are as follows (milligrams per liter of distilled, de-ionized water): $NaNO_3$, 22.5; KH_2PO_4, 0.15; K_2HPO_4, 7.5; $MgSO_4 \cdot 7H_2O$, 7.5; Na_2CO_3, 4.0; $CaCl_2$, 5.0; $Na_2SiO_3 \cdot H_2O$, 11.6; $FeCl_3 \cdot 6H_2O$, trace; $MnCl_2 \cdot 4H_2O$, trace; $ZnCl_2$, trace; $CoCl_2 \cdot 6H_2O$, trace; Na_2MoO_4, trace; H_3PO_3, trace; and $CuSO_4$, trace.

$^{239}Pu^{+4}$ Feed Solution. Twenty-five ml of 0.1 m ^{239}Pu solution (Los Alamos National Laboratory) tracer were transferred into a washed Pyrex centrifuge cone containing 1 ml of saturated $NaNO_3$ solution. The test tube was then placed in a heater block until the liquid had completely evaporated. The ^{239}Pu residue was resuspended in an appropriate amount of 0.1 N HCl and transferred to a washed polycarbonate Oak Ridge type 50-ml centrifuge tube to air dry. Filtered (0.05 μm) J-13 well water was used to resuspend the ^{239}Pu,

to a final concentration of 10^{-8} to 10^{-9} M ^{239}Pu. The oxidation state of the ^{239}Pu was predominantly +4, although some +5 and +6 may have also been present.

Sorption. The Pseudomonas sp. was transferred from a streak plate (nutrient agar, Difco Co.) to a 250-ml Belco culture flask containing 50 ml of nutrient broth (Difco). The flasks were incubated at room temperature for 18 h while shaking at 100 rpm. One ml of broth was transferred to another 250-ml flask containing 49 ml of nutrient broth. Again the culture was incubated at room temperature for 18 h. This 18-h culture was then used to inoculate fifteen, 250-ml Belco side-arm flasks each containing 49 ml of nutrient broth. The flasks were incubated at room temperature for periods of 3, 6, 12, 24, or 48 h. After each incubation period, three flasks were removed and the optical density of each culture was determined using a Klett-Summerson Photometer equipped with a #54 filter (520-580 nm transmission). The number of viable cell counts (or colony forming units) was then determined using standard-dilution and pour-plate methods (Standard Methods for the Examination of Water and Waste Water). The total broth volume from each side-arm flask was transferred to a 50-ml Oak-Ridge type, polycarbonate centrifuge tube and centrifuged at 17,300 x g for 10 min. The supernatant was discarded and the bacterial pellet was washed with 10 ml of phosphate buffer. The tubes were again centrifuged at 17,300 x g for 10 min. This process of washing the pellet was performed a total of three times. After the final washing, the supernatant was discarded and to each of the three tubes 20 ml of $^{239}Pu^{+4}$ feed solution was added. The tubes were agitated at 100 rpm at room temperature for 5 d. The tubes were centrifuged at 17,300 x g for 1 h, the supernatant was transferred to a 50-ml Oak Ridge type centrifuge tube and centrifuged again at 17,300 x g for 1 h. The pellets were allowed to dry in the air at

room temperature and weighed. Following the 1-h centrifugation, 10 ml of supernatant was transferred to another Oak Ridge type centrifuge tube and centrifuged at 17,300 x g for 2 h. Five ml of supernatant was removed and tranferred to a 20-ml scintillation vial to which 5 ml of water and 10 ml of Insta-Gel liquid scintillation cocktail (United Technologies, Packard Co.) were also added. The vials were mixed and placed in a Seale Mark II scintillation counter set at 50-min count time, window opening set at 1 to 99, maximum gain set at 000, the range switch set at F, and the scaler turned to the off position.

Sorption Ratio. The activity of each vial was determined three times. These values were averaged and the sorption ratio (activity in solid phase/activity liquid phase) was determined as follows: Background count was subtracted from the experimental count, yielding the activity in the liquid phase. The liquid phase activity was then subtracted from the activity of the feed solution, yielding the activity in the solid phase. The solid phase activity was multiplied by 4 (because only 5 of the initial 20 ml of feed solution was counted) and divided by the product of the dry weight of the bacteria times the activity in the liquid phase; i.e.,

$$\text{Sorption Ratio} = \frac{4 \times (\text{activity in the solid phase})}{(\text{dry weight}) \times (\text{activity in the liquid phase})}$$

Three replicates were performed for each experiment and each experiment was repeated three times. It should be mentioned that although our data are presented as a sorption ratio, there exists no information to demonstrate that sorption is actually occurring. What is being observed is the removal of plutonium from solution by the bacteria.

RESULTS

Bacterial Growth. Presented in Table I are the data for the optical densities, dry weights, and colony forming units of the bacteria. Each set of measurements demonstrates that the Pseudomonas sp. grew rapidly, reaching 2.1 x 10^9 cells ml^{-1} (or cells/ml) in 12 h of incubation. The cell numbers slowly increased to 3.4 x 10^9 cells ml^{-1} in 48 h. Clearly this species was able to maintain a high cell concentration over a prolonged time period.
Interestingly, when these cells were examined under a light microscope, a change in their morphology over time was observed. At 3, 6, and 12 h, the cells were rod shaped; however, after 18 h of incubation, the cells shrank in size and became coccoid in shape.

Sorption of $^{239}Pu^{+4}$ by the Pseudomonas. Presented in Table I are the results of the sorption experiments. Perhaps the most outstanding feature of the data is the strong sorption of the $^{239}Pu^{+4}$ by Pseudomonas. The bacteria concentrated the $^{239}Pu^{+4}$ nearly 1000 times greater than did the sterile control (1 g of sterile, crushed tuff, 75–200 mesh). Sorption also appears to be related to the physiological state of the bacteria. On a per-gram, dry-weight basis, the 3-h culture sorbed the most $^{239}Pu^{+4}$, followed closely by the 6-h cultures. The lowest sorption ratio was observed for the 12-h and

TABLE I. Optical density, dry weights, colony forming units (CFU) and sorption ratio for a Pseudomonas sp. over 48 h of incubation.

Incubation Time (h)	Optical Density (Klett Units)	Dry Weight (g)	CFU (per ml)	Sorption Ratio (ml/g)
3	7.53	0.00080	1.0×10^7	9,280 (707 sd)
6	26.8	0.00179	8.8×10^7	9,840 (4,417 sd)
12	174	0.01411	2.1×10^9	2,156 (161 sd)
24	173	0.01474	2.7×10^9	2,080 (230 sd)
48	155	0.01547	3.4×10^9	4,800 (342 sd)
Control (1 g crushed tuff)	—	1.000	—	5.37 (0.56 sd)

24-h cultures, followed by a slight increase in sorption by the 48 h cultures.

DISCUSSION

It is interesting that the classical log death phase of organism growth was never observed. Normally, one would anticipate that after 24 h, cell viability would begin to rapidly decrease, due to nutrient depletion and the build-up of toxic by-products. Rather, the bacteria increased in numbers throughout the duration of the experiment.

The observed shrinking of the bacteria is consistent with the finding of Humphrey et al.,[22] who have reported "dwarfism" in starving marine bacteria. The bacteria apparently enter this stage as a means of coping with a harsh

environment, and there may be a relationship between the decrease in size and the survival of the bacteria.

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Regardless of the means by which $^{239}Pu^{+4}$ is sorbed by the bacteria, the fact remains that these bacteria are able to remove this actinide from solution. What remains to be determined is the overall effect that this biological sorption may have on the movement of radioactive wastes away from a high level nuclear repository. It has been generally considered to be true that bacteria will be removed from suspension by soil or rock. In most classical saturated flow conditions, this is probably true; however, it is unclear what the effect the surrounding rock will have on the bacteria if saturated, fractur flow or unsaturated flow conditions are predominant. Secondly, it should always be kept in mind that most bacteria are motile, and therefore are not confined to an isolated location. Finally it must be emphasized that there exists little information regarding the extent of microbial activity in the deep, subsurface environment (Ghiorse, personal communication). It has always been assumed that microbial activity occurred only in the first few meters below the soil's surface. However, recent advances in sampling techniques have kindled new interest in the subject, and it is now known that significant microbial activity can occur to depths of 30 m or more below the surface. Unfortunately, these sampling techniques are not able to access microbial activity at greater depths; therefore, the extent of microbial activity at the depths of the candidate high level nuclear waste repository is still subject to considerable conjecture.

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TRANSPORT BY MICROORGANISMS:

CHELATION

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Abstract

Siderophores are low-molecular-weight compounds produced by microorganisms. These compounds are used by microorganisms to solubilize the very insoluble Fe^{3+} cation. Because of the atomic similarities of Pu^{4+} and Fe^{3+}, it may be possible that siderophores could bind Pu^{4+}. The chelation of Pu^{4+} by siderophores may increase its transport, and the transport of other actinides, away from the candidate high-level nuclear waste repository at Yucca Mountain. The binding of Pu^{4+} is being investigated in our laboratory and is the subject of this report. Preliminary results indicate that the siderophore produced by a Pseudomonas sp. does bind to Pu^{4+}. Experiments are now being performed to determine the formation constant of the Pu^{4+}/siderophore complex. This information will be incorporated into the sorption studies now being performed at Los Alamos.

1.0 Introduction

During the process of characterization, by the Nevada Nuclear Waste Storage Investigations (NNWSI) Project, of the geology and hydrology of the site for a candidate high-level nuclear waste repository at Yucca Mountain, Nevada, several million gallons of drilling fluids were used. Within a 3-km radius of the proposed site of the Exploratory Shaft Facility (ESF) it is estimated that nearly 15,000,000 gallons of drilling fluids were released into the block (candidate site) or in the immediate area. The drilling logs from these wells indicate that a significant amount of the fluid was lost in the Topopah Spring Member, which would be the geologic location of the repository. The Topopah Spring Member has been characterized as a welded tuff with a low permeability and a high content of fractures. Montazer and Wilson (1) have therefore suggested that in this member lateral movement of water may exceed downward rates, a statement that was substantiated by the recent discovery of drilling fluids in the USW UZ-1 hole (air-drilled without fluids). Interestingly, UZ-1 is located several hundred meters from the closest fluid-drilled holes (USW G-1 and H-1), and its drilling was limited to the unsaturated zone, at a depth approximately equal to that of the repository.

In addition to those drilling fluids, several other forms of organic materials

will be introduced into the block. During the construction of the exploratory shaft, items such as diesel fuels and exhausts, hydraulic fluids, and additional drilling fluids will be discarded routinely. It can be anticipated that many of the same materials will be used during the construction of the repository as well. Therefore, before the repository is sealed there will be a significant amount of organic material located near it. A comprehensive inventory of the fluids that are going to be used during the construction and operation of the ESF is now being prepared at LANL (2).

Our specific concern is that these fluids and organic materials may be utilized as growth substrates by a large number of microorganisms, which in turn may influence the transport of radioactive elements away from the repository. Microorganisms can affect transport in one or more of the following ways:

1) alter the composition of the groundwater chemistry through changes in pH or Eh,
2) retard the transport of the radionuclide by sorption onto a nonmotile solid phase,
3) transport the radionuclide via biological movement,
4) transport the radionuclide via colloidal dispersion,
5) solubilize radioactive elements by producing chelating agents.

In this paper we report experiments designed to study the chelation of actinide elements.

A very important principle of microbial ecology is that microorganisms are able to strongly influence the movement of metals through soils. One example is the microbial production of powerful chelating agents called siderophores that are used to solubilize the very insoluble Fe^{3+} cation. In the last three decades over 80 siderophores have been isolated and characterized. They have formation constants for Fe^{3+} as high as 10^{52}. At pH 7 the equilibrium concentration for ferric ion (as free Fe^{3+}) is approximately 10^{-18} M. For microorganisms such as enteric bacteria, which need at least a total iron concentration of 5.0×10^{-7} M for optimal growth, that concentration (10^{-18} M) is many orders of magnitude too low. Only powerful chelating agents such as the siderophores can mobilize iron from the environment and facilitate its transport into the microbial cell.

Neilands (3) has described a siderophore as a "low-molecular-weight (500–1000 daltons) virtually ferric-specific ligand, the biosynthesis of which is carefully regulated by iron and the function of which is to supply iron to the cell." As iron is involved in several critical stages in metabolism, microbes have evolved multiple systems for its acquisition. The high affinity system is composed of the siderophores and the matching membrane-associated receptors (4); only the former component of the high affinity system is discussed in this paper. Siderophores are viewed as the evolutionary response to the appearance of O_2 in the atmosphere, the concomitant oxidation of Fe^{2+} to Fe^{3+}, and the precipitation of the latter as ferric hydroxide (5). This explanation accounts for the general occurrence of siderophores in aerobic and facultative anaerobic (able to grow under both aerobic and anaerobic conditions) microorganisms (6). Ferric siderophores are octahedral complexes in which the coordinated metal ion is d^5, high-spin electronic configuration, and rapidly exchangeable. Generally siderophore ligands are considered to be "hard" bases, and therefore show little affinity for the "softer" acid cations, including Fe^{2+}, providing a means of release of iron via a reduction step. The single most outstanding feature of the siderophores is their extremely high affinity for the ferric ion (7).

Neilands (3) believes that because Fe^{3+} and Pu^{4+} are similar in their respective charge/ionic-radius ratio (4.6 and 4.2 respectively), Pu^{4+} may possibly serve as an analog to Fe^{3+}. In fact, Raymond and coworkers (8) have successfully synthesized a series of siderophore-like tetracatecholate ligands with both linear and cyclic tetramines as backbones. The eight-coordinate nature of Pu^{4+} is well satisfied by the four catecholate moieties, resulting in an effective binding of the Pu^{4+}. One could therefore assume that a siderophore would bind to Pu^{4+} with approximately the same formation constant as that with Fe^{3+}. The relevance of this information to the proposed high-level nuclear waste repository is quite significant, because a large population of soil microorganisms having a strong metabolic need for iron could co-metabolically chelate the plus four oxidation-state actinides. If the bacterial population is large enough (provided with a large energy source), then it is possible that radioactive elements could be chelated, resulting in the solubilization of the actinide element. Therefore, the net effect of chelation would be an increase in the transport of the actinide

elements through the aqueous phase found at Yucca Mountain. A purpose of the microbiology subtask[15] to estimate the potential for solubilization via chelation and to predict the significance of this effect as it applies to the performance of the repository.

2.0 Materials and Methods

The medium used for most of the experiments consisted of ($g \cdot L^{-1}$): K_2HPO_4, 6 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 3H_2O$, 2 g; succinic acid, 4 g; pH = 7.0. Iron was removed by passing all of the constituents of the medium through a Chelex 100 column. The concentration of iron was adjusted to 1.0 mM. For large preparations, cultures were incubated at room temperature (22°C), while smaller preparations were incubated at 36°C. The presence of the siderophore was detected by the addition of an equal volume of $Fe(ClO_4)_3$ (5.0 mM $FeCl_3$ in 0.14 M $HClO_4$) to 1 ml of the spent medium. The sample was then centrifuged at 1800 g for 5 min, and a deep amber color in the supernatant indicated the presence of the siderophore.

Three 2-L batches of succinate medium were inoculated with a 24-h culture of a Pseudomonas sp. (previously isolated from the Nevada Test Site) and incubated at room temperature, under constant agitation. After 40 h of incubation the cultures were centrifuged (4000 g, 30 min at 4°C) and the cell-free supernatant was evaporated under reduced pressure to approximately 100 mL. The concentrated material was then extracted with an equal volume of chloroform: phenol solution (1:1, v/v). The organic phase was treated with an equal volume of water and ether. The aqueous phase of this second partition was extracted two additional times with an equal volume of ether. The aqueous phase of the final partition was further concentrated to about 30 mL. This concentrated aqueous phase was loaded onto a CM-Sephadex C-25 column and eluted with 0.1 M pyridine/acetic acid (pH = 6.5; flow rate, 20 drops per min). Ten-milliliter fractions were collected and absorbance was measured at 280 nm (Beckman Spectrophotometer, Beckman Instruments, Inc.). The fractions containing the siderophore were further purified on a Bio-Gel P2 column and eluted with glass-distilled water.

Protein Assay. A modification of Lowry's method was utilized:

1. sample + dd H_2O was brought to 1 mL, to which was added 50 μL of 0.15% deoxycolate and 100 μL of 72% TCA;
2. it was centrifuged at 11,000 g for 30 min at 4°C;
3. 1 mL of dd H_2O was added to the pellets and mixed well;
4. reagent A* (1.0 mL) was added, mixed well, and incubated at room temperature for 10 min;
5. reagent B** (0.5 mL) was added, mixed well, incubated for 30 min; and
6. the absorbance was read at 750 nm.

*Reagent A = 1 vol copper-tartarate carbonate
1 vol 0.8 N NaOH
1 vol 10% SDS
1 vol dd H_2O

**Reagent B = 1 vol Folin (2N)
1 vol dd H_2O

Siderophore Assay. The presence of siderophore was indicated by the presence of a deep amber color upon the addition of $FeCl_3$ [later changed to $Fe(ClO_4)_3$] to the spent medium. For the uninoculated controls no color change was observed. This deep amber color also suggests the presence of a hydroxamate-type siderophore, as opposed to a catecholate-type, because according to Neilands (3) and Emery (9), upon addition of ferric iron to spent medium, trishydroxamates are orange colored, while triscatacholates are wine colored.

Siderophore Isolation and Purification. Three peaks collected from the CM-Sephadex C-25 column were observed at 280 nm. The first and third peaks usually complexed with $Fe(ClO_4)_3$, displaying a deep color change while the second peak showed less of a color change. The fractions containing the siderophore were freeze-dried and stored in sealed plastic test tubes.

High-Pressure Liquid Chromotography (HPLC) Purification. Approximately 1 mg of freeze-dried siderophore from each of the three CM-Sephadex C-25 fractions was/were reconstituted in 200 μL of HPLC-grade water and injected into a Waters

model 590 HPLC equipped with a C-18 column (flow rate, 2 $mL \cdot min^{-1}$; attenuation, 1.0; absorbance, 206 nm). The solvent gradient was 15 min of isocratic flow (H_2O), and 1-h linear gradient, from 100% solvent A (100% H_2O) to 100% solvent B (50% H_2O, 50% acetonitrile). For the first of the Sephadex fraction, major peaks were detected at approximately 2.0 and 24-45 min (Fig 1a). The second Sepadex fraction contained four major HPLC peaks at approximately 2.5, 6.0, 14.0, and 22.0 min (Fig. 1b). The third Sephadex fraction contained only one peak at 4.0 min (Fig 1c). Each of these peaks has been tested for siderophore content using the $FeCl_3$ [has not been repeated using $Fe(ClO_4)_3$], and for the first and third Sephadex fractions most of the siderophore was found in the early (2.0 and 4.0 min, respectively) peak (Fig. 2a and 2b). Very little siderophore was detected in the second Sephadex fraction .

Formation Constant with ^{239}Pu. In order to evaluate the significance of chelation, it is necessary to determine the formation constant of the siderophore/actinide complex. Ideally we prefer to work at neutral pH, in order to remain consistent with environmental conditions found at Yucca Mountain. For $^{239}Pu^{4+}$ the best way to avoid spontaneous complexation at neutrality is to work with very dilute solutions, such as 10^{-8} M or lower. Classically these solutions have been prepared by diluting an acidic $^{239}Pu^{4+}$ feed solution. Hobart (10) is currently using carbonate complexation to stabilize the oxidation state of the actinide. Regardless of the preparation method, the plutonium ion must be diluted to avoid complexation. However, because of the low actinide concentration, classical spectrophotometric and electrochemical analysis of siderophore/$^{239}Pu^{4+}$ complexation cannot be used to determine the formation constant of the complex.

For that reason we chose to use competition experiments between the siderophore and a known metal-chelating agent to measure formation constants. Chelex 100 is a chelating ion exchange resin which shows unusually high preference for copper, iron, and other heavy metals. It is a styrene-divinyl benzene copolymer matrix to which iminodiacetic acid residues are attached, hence its selectivity for metals corresponds to that of iminodiacetic acid. Raymond and coworkers (8) have described the atomic similarities of Fe^{3+} and Pu^{4+}. It is therefore appropriate to assume that Chelex 100 will bind to Pu^{4+}

in a manner analogous to that of Fe^{3+}, and therefore can be used to determine the formation constant between siderophore and $^{239}Pu^{4+}$.

Experiments were first performed to test the feasibility of using Chelex 100 in competition experiments. Competition among Chelex 100 and ethylenediaminetetraacetic acid (EDTA) for ferric iron was tested. The preparation of the experiment was as follows:

1) made up 3 mL total volume of seven different EDTA solutions;
2) transferred 0.2 mL of 2.0, 1.0, 0.5, 0.2, 0.1. .05, and .02 M EDTA into 3.8 mL of water;
3) divided the 4.0 mL volume of each in half;
4) to one-half added 1 mL of saturated Chelex 100 sediment [chelex beads saturated with $Fe(ClO_4)_3$]; and
5) to the other half added 1 mL of saturated Chelex 100 water (supernatant).

The final concentrations of EDTA in each of the reaction vessels (15-mL plastic, Pyrex centrifuge tubes) were 6.67×10^{-2} μM, 3.33×10^{-2} μM, 1.67×10^{-2} μM, 6.67×10^{-3} μM, 3.33×10^{-3} μM, 1.67×10^{-3} μM, and 6.67×10^{-4} μM. The centrifuge tubes were agitated for 10 h at room temperature. Two milliters of fluid was removed from each tube, and adsorbance was measured on a Perkin-Elmer model 552 scanning spectrophotometer, using the corresponding saturated chelex water supernatant as a blank. This experiment was performed in triplicate and the results are presented in Fig. 3.

A similar experiment was performed using siderophore instead of EDTA. The purpose of this experiment was to determine if siderophore would compete with Chelex 100 for ferric iron. The concentration of siderophore was adjusted to approximately 0.014 M, based upon the assumption that the molecular weight of the siderophore is approximately 1000 daltons. Although we have no direct evidence that the molecular weight of the siderophore is that value, this approximation was based on size exclusion using molecular sieves. The siderophore passed through a 2000 sieve, while being retained by a 500-molecular-weight sieve. The volume of chelex added was either 5.0, 25, 125, 625, or 1000 μL (bed volume). The results of this experiment are presented in

Fig. 4.

Experiments were then performed to determine the feasibility of using competition experiments with $^{239}Pu^{4+}$. One milligram of siderophore was reconstituted in 2 mL of triple-distilled deionized water. Solutions of 5, 50, or 500 μL were added to 10 mL of triple-distilled deionized water which contained 50 μL of washed, deionized Chelex-100 beads (Bio Rad) having an exchange capacity of 0.4 meq mL^{-1}. To these solutions 0.1 mL of approximately 10^{-6} to 10^{-7} M $^{239}Pu^{4+}$ feed solution was added and mixed for 24 h at room temperature. The Chelex 100 beads were removed by centrifugation and 5 mL of supernatant was placed in liquid scintillation vials, to which 5 mL of filtered J-13 water (0.05 Nucleopore) and 10 mL of Insta Gel scintillation flour were added. Activity in those tubes containing both siderophore and Chelex 100 was compared with activity in those tubes containing only Chelex 100.

3.0 Results and Discussion

Siderophore Assay. Although siderophores have been isolated for over 80 species of microorganisms, little is known about these compounds. This lack of information tends to be uniform throughout all phases of siderophore research. Therefore, much of the work done by the NNWSI microbiology subtask has been the development of procedures. Often we found that a procedure, or part of a procedure, yielded conflicting results. The use of $FeCl_3$ in the assay procedure yielded such results. Sometimes a fraction (from the C-25 column) that was suspected of containing the siderophore would test positive upon the addition of $FeCl_3$, or sometimes it would test negative. At first it was assumed that the siderophore was not really present in the negative fractions and that some other part of the procedure was at fault (e.g., growth conditions). However, after extensive experimentation it was determined that the problem was the nonspecific reaction of $FeCl_3$ with other media components that often resulted in flocculation, thereby interfering with the spectrophotometric measurements. Alternative compounds such as $Fe(NH_4)_2C_6H_7O_7$), FeO_3, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $Fe_2(SO_4)_3 \cdot xH_2O$, $Fe(NO_3)_2 \cdot 9H_2O$, and

$Fe(ClO_4)_3$ were all tested for use in the assay. Ferric perchlorate [$Fe(ClO_4)_3$] was determined to give the best results and therefore was used in subsequent assays.

Concentration of Iron. As stated in the introduction, the production of siderophore by microorganisms is strictly regulated by the presence of iron. Normally one induces the production of siderophore by reducing the amount of available iron; however, one has to be careful, because iron is a requirement for growth, and if iron becomes limiting then microbial growth can become impaired. If that happens, then overall siderophore production could become reduced. The key, therefore, is to achieve maximum bacterial growth prior to achieving maximum sideorphore production, both of which should occur before the cells reach stationary phase. In other words, the cells should be supplied with an initial concentration of iron that allows them to enter log growth phase. Once the cells have entered log growth phase, the concentration of iron becomes limiting and results in the induction of siderophore production in a maximum number of cells. In order to achieve these results, one must first remove all of the iron from the growth medium and then add a known concentration of iron.

The removal of iron was accomplished by passing stock solutions of each of the salts, and the water, used in the growth medium through a column of Chelex 100 ion exchange beads. Then a series of simple experiments were performed to determine what concentration of added iron resulted in the greatest production of siderophore. Iron was added to iron-free growth medium to obtain concentrations of either 0.1, 1.0, 10, or 100 μM, and this experiment was repeated six times. Even though the results varied among the experiments, those flasks containing the 1.0-μM concentration of iron most often yielded the highest concentration of siderophore. Therefore, in subsequent experiments, the final concentration of iron was adjusted to 1.0 μM.

HPLC Analysis. Using HPLC analysis it became immediately obvious that the siderophore was either not pure or that there was more than one siderophore present in each of the peaks collected from the C-25 Sephadex column (Figs. 1a, 1b, 1c).

Three different C-25 column peaks have been analyzed by the HPLC purification method, and in each case it was the early fractions that tested positive with the iron assay (Figs. 2a, 2b). It is unclear why more than one C-25 Sephadex fraction tested positive for siderophore with the iron assay. Perhaps the Pseudomonas sp. was producing more than one siderophore, or perhaps a single siderophore was breaking down into several components.

It is not uncommon for a microorganism to produce more than one siderophore (11). Pseudomonas aeruginosa is known to produce pyochelin and pyoverdin, Escherichia coli has been found to produce both enterocholin and aerobactin, and Azotobacter vinelandii produces dihydroxybenzoic acid, azotochelin, and azotobactin. Siderophore synthesis is controlled in a sequential manner, and is governed by the increasing severity of iron limitation (12). Organisms appear to conserve energy while synthesizing siderophores, expending energy for the synthesis of the most complex, but most effective, siderophore only when it is essential to do so. However, in a recent investigation of the production of pyroverdin$_{pf}$ and ferribactin by Pseudomonas fluorescens it was found that ferribactin was produced late in the culture, even though pyoverdin$_{pf}$ was thought to be the most effective of the two (13, 14, 15). Therefore, sequential synthesis dependent upon iron demand is not known to exist for these siderophores, but in all of these examples it is believed that multiple siderophores are produced because one of the siderophores is critical for growth under severe iron deprivation. Based upon this information, it would not be surprising to find that the Pseudomonas sp. used in our studies may be producing more than one siderophore.

A second possible explanation of the multiple C-25 Sephadex peaks is that degradation of the siderophore could be occurring. The microorganism that we have been working with is an unknown Pseudomonas sp., isolated from the NTS, and closely resembles a P. stutzeri. Due to its unknown identity, we could assume that many of its metabolic products may be unique, including siderophore. The methods that we have used to isolate and purify siderophore have been modifications of procedures taken from the literature. Because these methods were not developed specifically for this Pseudomonas sp., it is possible that a method, or part of the method, may be causing a partial degradation of the siderophore. As a precaution we are continually improving

these procedures, while reviewing the literature for better methods.

Formation Constant. One of the objectives of the chelation research project is to determine the formation constant between the siderophore and actinide elements. Once this information is obtained, then we can make a more meaningful assessment of the role that microorganisms play in the transport of actinide elements through tuffaceous rock. Preliminary experiments were designed to determine the feasibility of using competition between the ligand and Chelex 100 for the metal ion.

EDTA is a chelating ligand having a strong preference for divalent cations, and it clearly competed with Chelex 100 for ferric iron, as seen in Fig. 3. With increasing concentrations of EDTA, the adsorbance of the EDTA/Fe^{3+} complex increased significantly. Evidently the EDTA was removing the Fe^{3+} ion from the saturated Chelex 100 resin.

The results of the siderophore competition experiments show a similiar trend. With increasing concentrations of Chelex 100, the adsorbance maximum of the siderophore/Fe^{3+} complex significantly decreased (Fig. 4). Therefore, we can assume that the Chelex 100 was removing the ferric ion from the siderophore. However, it is important to point out that in this experiment the iron was added to the siderophore first and not to the Chelex 100. When the iron was added to the Chelex 100 first, as was done in the EDTA experiment, no siderophore/iron adsorption could be observed. Yet, when the iron and siderophore were mixed together, it was possible to observe a decrease in the siderophore/Fe^{3+} complex adsorbance. Obviously the Chelex 100 was able to remove ferric iron from the siderophore, but the siderophore was unable to remove ferric iron from the Chelex 100. At this time we are unable to explain this anomaly.

The final set of preliminary experiments demonstrated that competition between siderophore and Chelex 100 for $^{239}Pu^{4+}$ may prove to be a useful technique for calculating the formation constant between siderophore and actinide elements. Although this experiment was performed only once, and crude siderophore preparations were used, the results demonstrated that at a higher amount of Chelex 100 less $^{239}Pu^{4+}$ was bound to the siderophore. It appears that the

siderophore does bind to $^{239}Pu^{4+}$ and that the formation constant between siderophore and $^{239}Pu^{4+}$ is similar to that of Chelex 100 and $^{239}Pu^{4+}$.

4.0 Conclusions

The progress of the chelation experiments is very interesting. An unknown microorganism, isolated from the NTS, has been found to strongly bind to $^{239}Pu^{4+}$. This species is known to produce powerful metal-binding ligands, called siderophores. These siderophores have been isolated and are now being purified in the laboratory. Preliminary experiments suggest that the siderophore does bind to $^{239}Pu^{4+}$. Experiments are now being conducted to determine the formation constant of the siderophore/$^{239}Pu^{4+}$ complex. The information generated from these experiments will be used to predict the movement of actinide elements through the tuffaceous rock found at Yucca Mountain, as influenced by microorganisms. This information will be incorporated into the sorption studies now being performed at Los Alamos.

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Figures

Figure 1a. HPLC chromatograph of fraction 17-1 taken from a Sephadex column.

Figure 1b. HPLC chromatograph of fraction 17-2 taken from a Sephadex column.

Figure 1c. HPLC chromatograph of fraction 17-3 taken from a Sephadex column.

Figure 2a. Adsorption maximums of fractions 1 and 2 [plus $Fe(ClO_4)_3$] taken from HPLC run 17-1 (figure 1a).

Figure 2b. Analysis of fraction from figure 1c for siderphore.

Figure 3. Adsorption (280 nm) vs EDTA concentration in the presence of Chelex 100 [plus $Fe(ClO_4)_3$].

Figure 4. Adsorption (295 nm) vs Chelex in the presence of siderophore [plus $Fe(ClO_4)_3$].

FIG. 1A

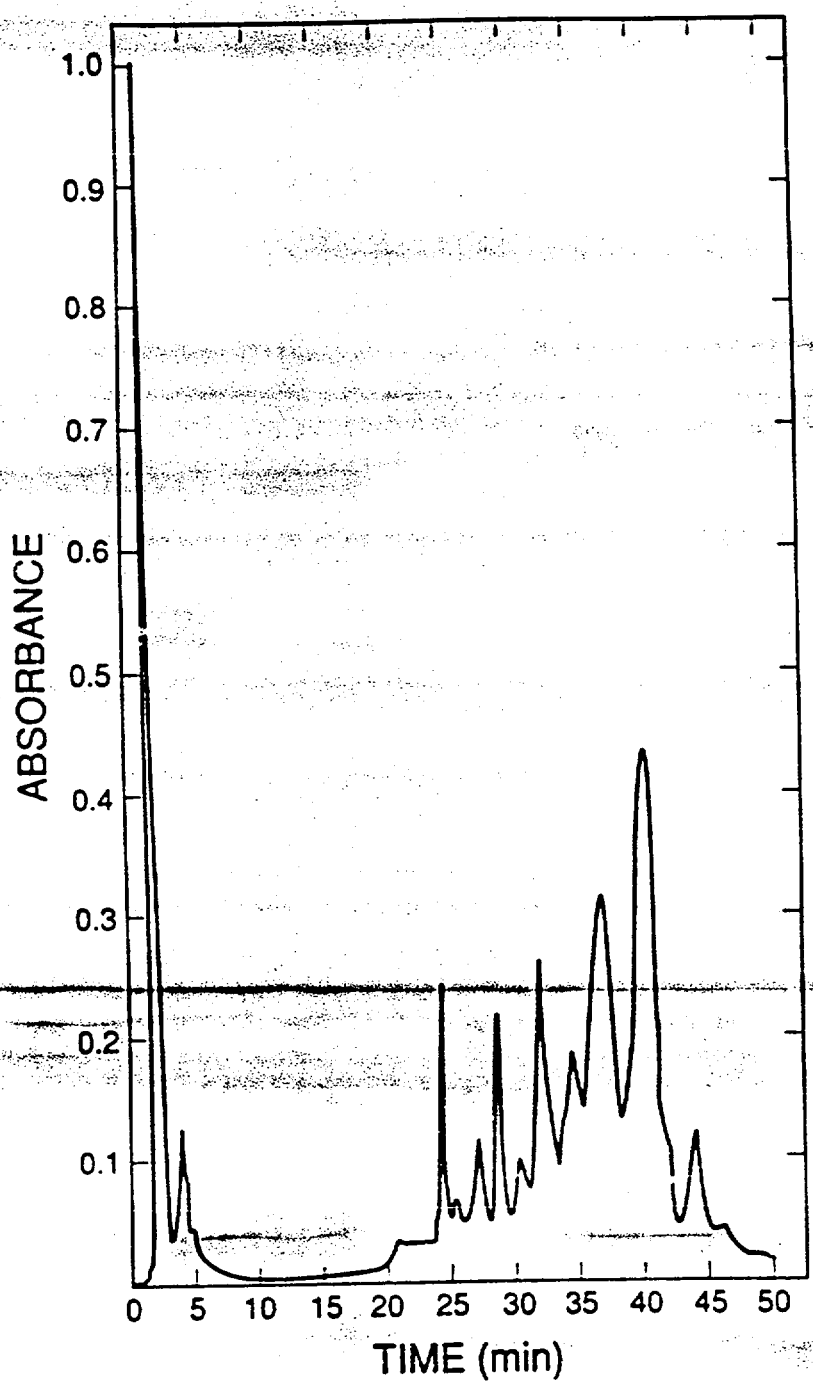


FIG. 1B

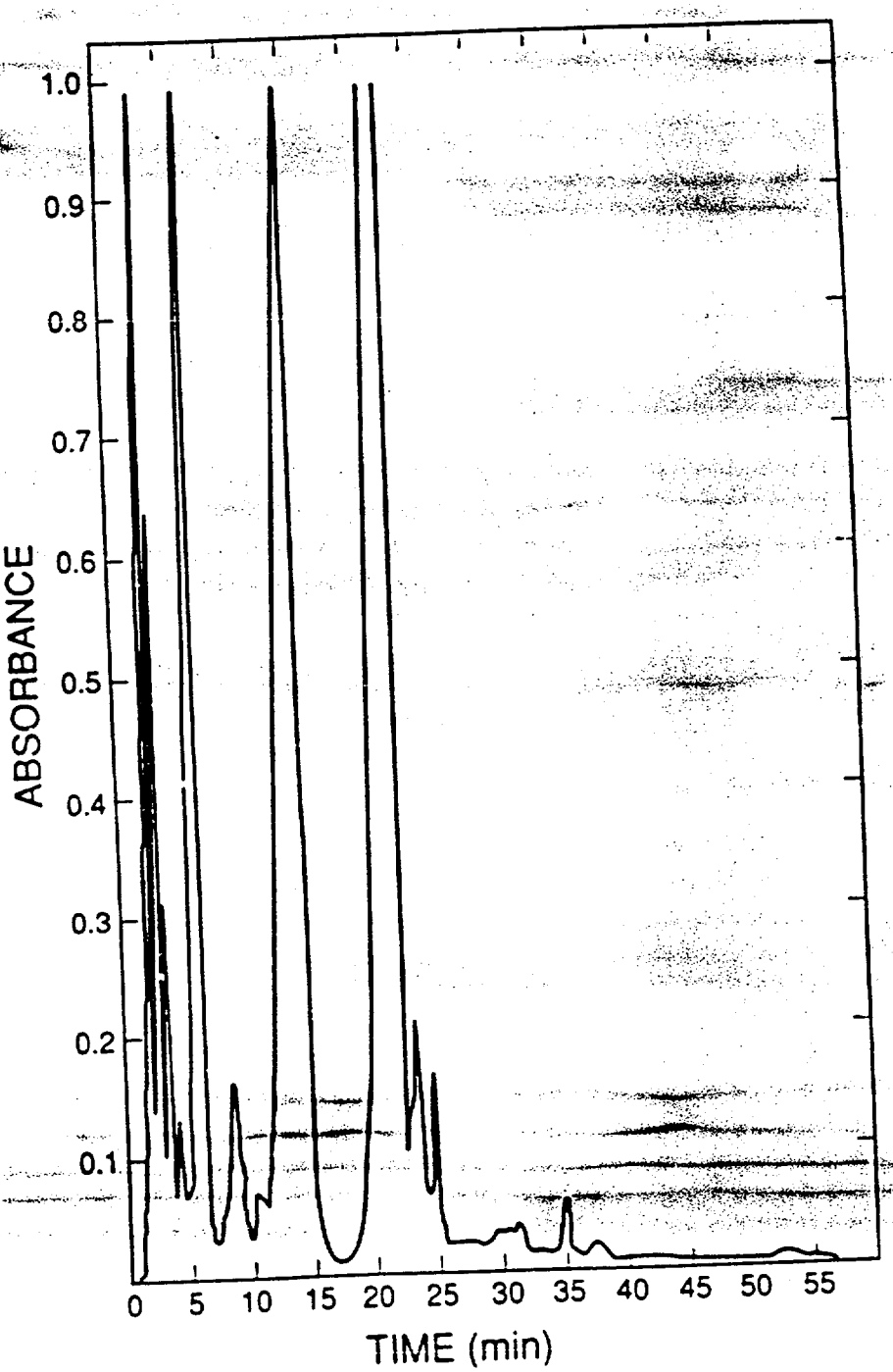


FIG. 1C

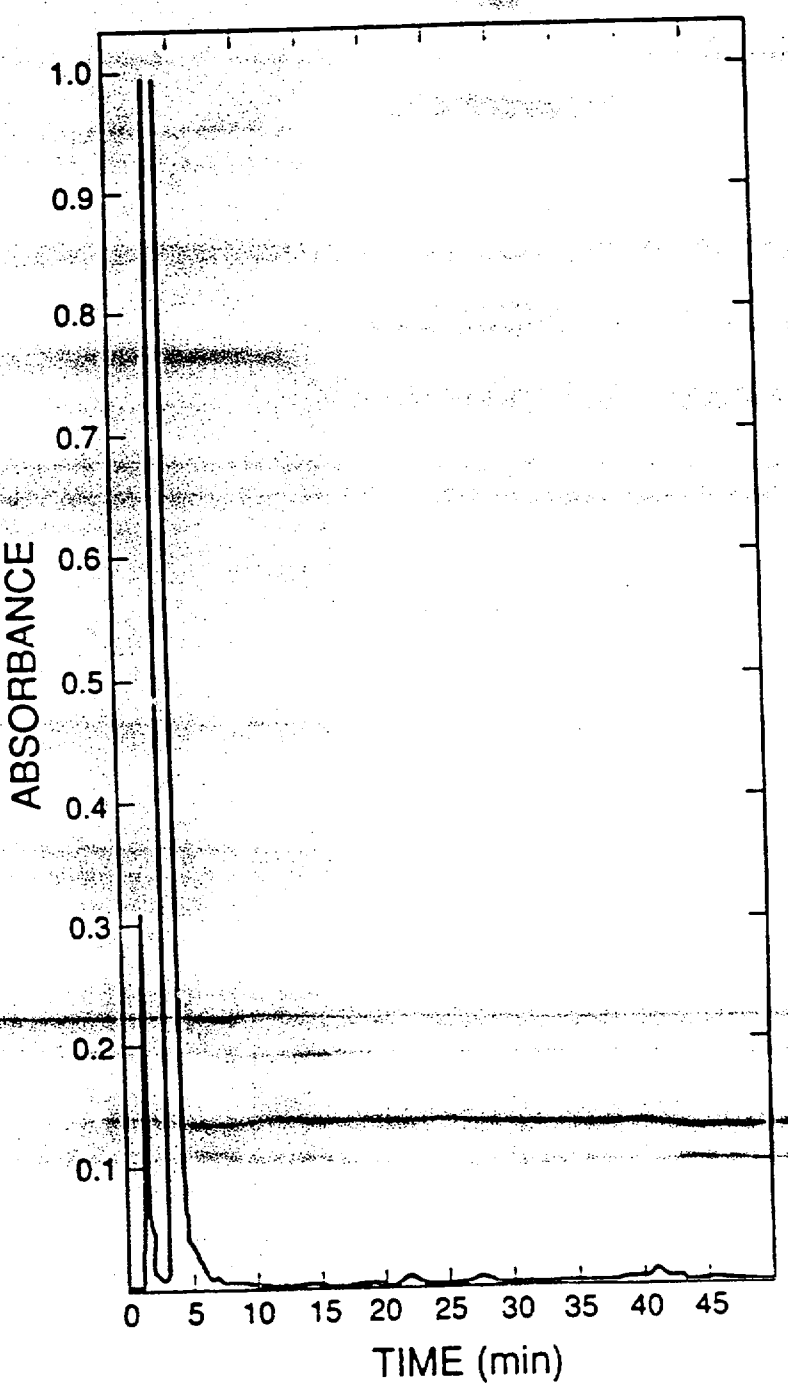


FIG. 2A

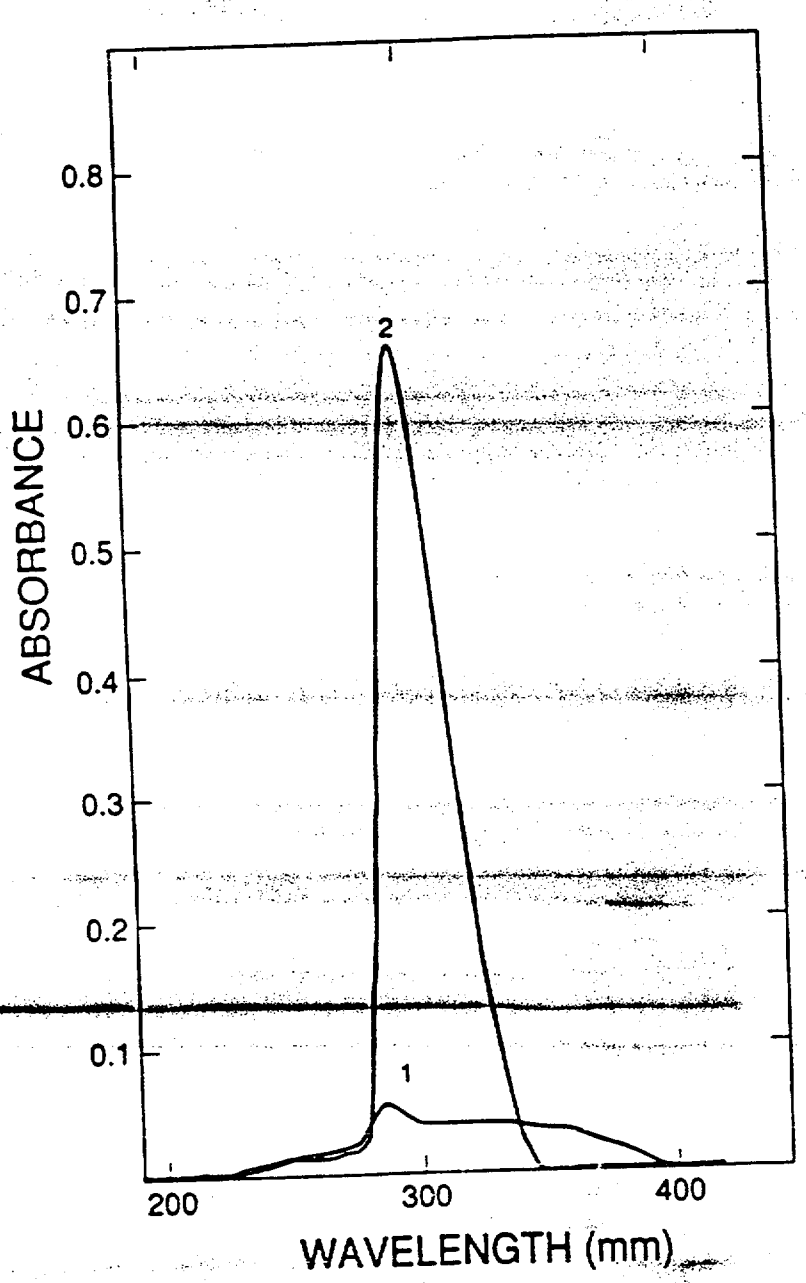


FIG. 2B

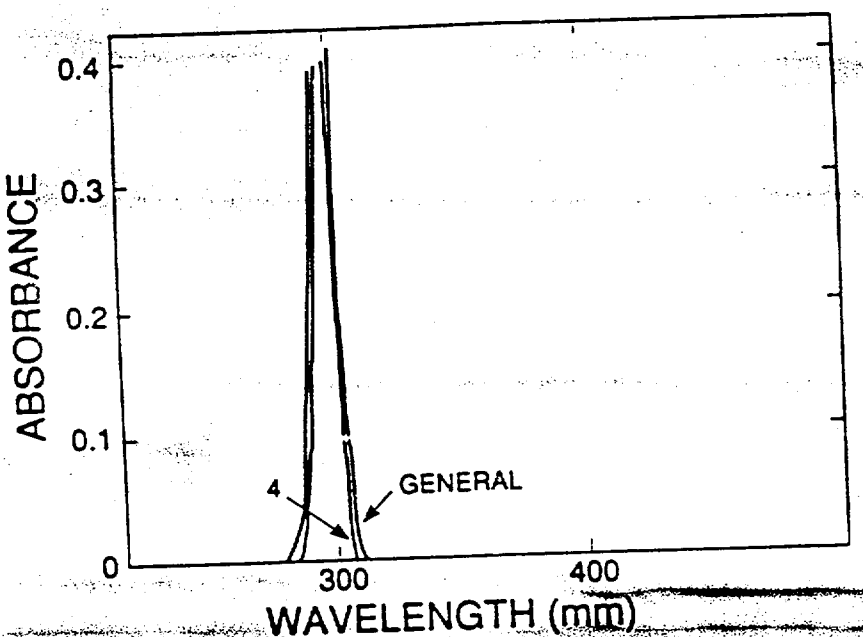


FIG. 3

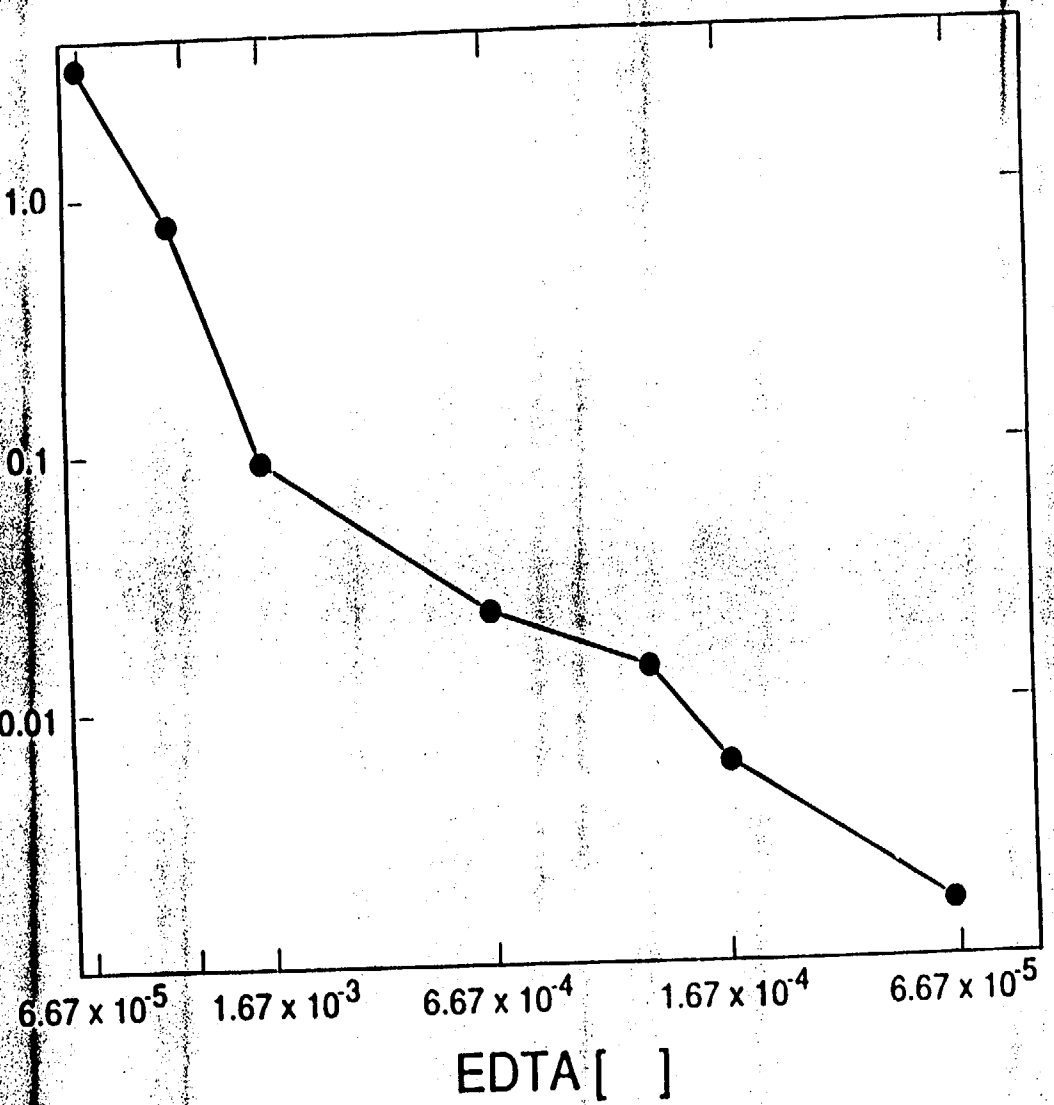
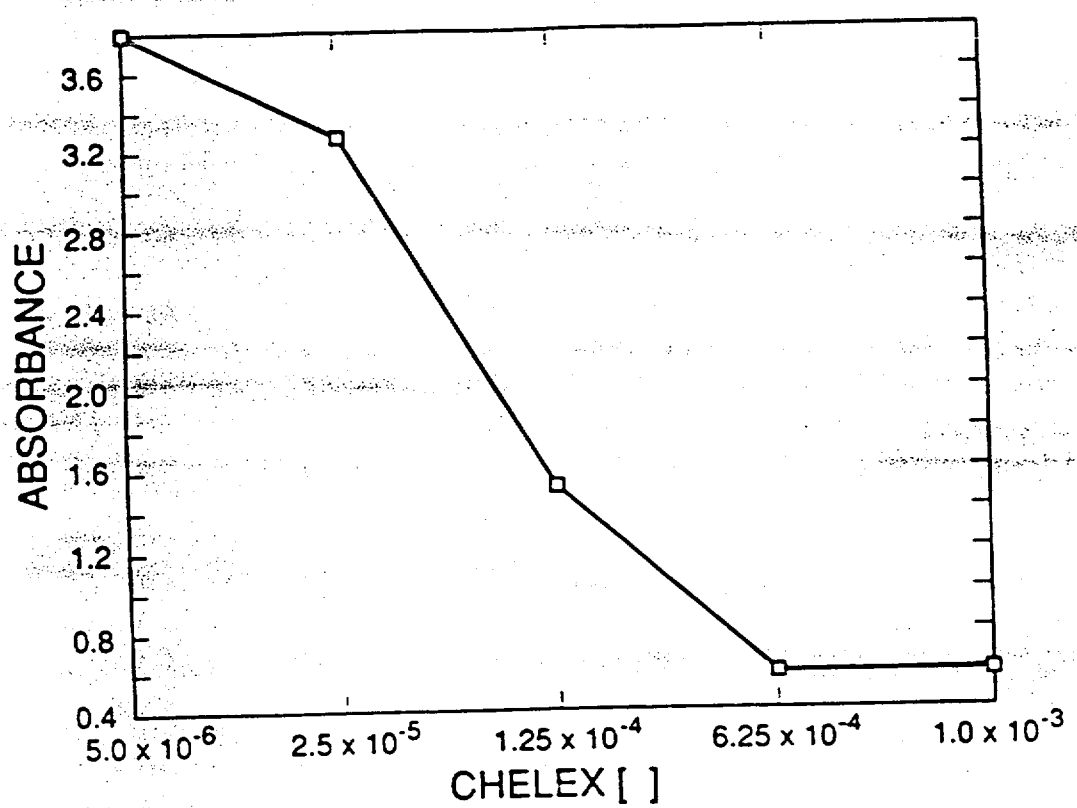

ABSORBANCE
1.0
0.1
0.01
6.67 x 10-5
1.67 x 10-3
6.67 x 10-4
1.67 x 10-4
6.67 x 10-5
EDTA [ ]

FIG. 4



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TRANSPORT BY MICROORGANISMS:

COLLOIDS

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Abstract

Colloids are suspected to play an important role in the dispersion of toxic wastes, heavy metal contaminants, and radioactive wastes in the subsurface environment. Preliminary studies performed at Los Alamos National Laboratory suggest that the presence of microorganisms significantly accelerates the formation of colloidal agglomerates. Within 50 hours, nearly all single colloids had formed multi-particle agglomerates. The experiments presented describe the role that microorganisms play in the formation of colloidal agglomerates and how this interaction affects the transport of radioactive wastes.

1.0 Introduction

During the process of characterization, by the Nevada Nuclear Waste Storage Investigations (NNWSI) Project, of the geology and hydrology of the site for a candidate high-level nuclear waste repository at Yucca Mountain, Nevada, several million gallons of drilling fluids were used. Within a 3-km radius of the exploratory shaft it is estimated that nearly 15,000,000 gallons of drilling fluids were released into the block (candidate site) or in the immediate area.

The drilling logs from these wells indicate that a significant amount of the fluid was lost in the Topopah Spring Member, which would be the geologic location of the repository. The Topopah Spring Member has been characterized as a welded tuff with a low permeability and a high content of fractures. Montazer and Wilson (1) have therefore suggested that in this member lateral movement of water may exceed downward rates, a statement that was substantiated by the recent discovery of drilling fluids in the USW UZ-1 hole (air-drilled without fluids). Interestingly, UZ-1 is located several hundred meters from the closest fluid-drilled holes (USW G-1 and H-1), and its drilling was limited to the unsaturated zone, at a depth approximately equal to that of the repository. In addition to those drilling fluids, several other forms of organic materials will be introduced into the block. During the construction of the Exploratory Shaft Facility (ESF), items such as diesel fuels and exhausts, hydraulic fluids, and additional drilling fluids will be

discarded routinely. It can be anticipated that many of the same materials will be used during the construction of the repository as well. Therefore, before the repository is sealed there will be a significant amount of organic material located near it. A comprehensive inventory of the fluids that are going to be used during the construction and operation of the ESF is now being prepared at LANL (2).

Our specific concern is that these fluids and organic materials may be utilized as growth substrates by a large number of microorganisms, which in turn may influence the transport of radioactive elements away from the repository. Microorganisms can affect transport in one or more of the following ways:

1) alter the composition of the groundwater chemistry through changes in pH or Eh,
2) retard the transport of the radionuclide by sorption onto a nonmotile solid phase,
3) transport the radionuclide by biological movement,
4) transport the radionuclide by colloidal dispersion,
5) solubilize radioactive elements by producing chelating agents.

In this paper the progress of experiments designed to study colloidal dispersion will be discussed.

Colloidal dispersion has been implicated as a means of transporting toxic wastes, heavy metals, and radioactive wastes through soil and rock systems (3; J. McCarthy, Oak Ridge National Laboratory, personal communication). When attached to a colloid, these substances are unable to participate in adsorption/desorption reactions with the soil or rock matrix. These substances then move at an accelerated rate with the colloids though the soil or rock matrix. If, however, colloids become attached to one another to form agglomerates, then these particles would no longer be available to participate in colloidal dispersion processes, because of size exclusion. Obviously, with increased agglomerate size, the effect of size exclusion would also increase, and the net result would be an overall decrease in the transport of radioactive wastes. With that supposition we investigated the influence of bacteria upon the agglomeration of clay colloids.

It would be worthwhile to first review what is known regarding the interactions between microorganisms and colloidal particles. Those interactions include the attraction processes, adhesion, adsorption, the effects of colloids on microbial metabolism, and flocculation.

1.1 Attraction of Bacteria to Solid Surfaces

Since many natural habitats have a low nutrient status, solid surfaces are potential sites for concentration of nutrients (as ions and macromolecules) and, consequently, of intensified microbial activity. The movement of water across a surface provides increased opportunities for microorganisms to approach solid-liquid interfaces. In addition, there are many physicochemical and biological attraction mechanisms operative in the immediate vicinity of interfaces:

1) Chemotaxis. Mobile bacteria are capable of a chemotactic response to low concentrations of nutrients introduced into a normally nutrient-deficient system. Chemotaxis, of course, cannot account for the attraction of nonmotile bacteria to solid-liquid or other interfaces.
2) Brownian motion. Colloidal particles are in a state of continual random motion (Brownian motion) caused by the chaotic thermal motion of molecules that collide with each other and with particles suspended in the liquid;
3) Electrostatic attraction. The interaction of negatively charged surfaces of bacteria with solid surfaces probably depends on the properties of the surface in question. Because most surfaces in nature are negatively charged, it is unlikely that electrostatic phenomena are involved directly in the attraction of bacteria to such surfaces. It should be emphasized, however, that solids in natural environments often acquire different surface properties through

sorption of macromolecule compounds to the surface. Therefore, the surface, as "seen" by the bacteria, may be very different from the original surface.

4) Electrical double layer effects. When two negatively charged bodies are in close association, they may be repelled from or attracted to each other, an effect that depends on the thickness of the interacting electrical double layers, which, in turn, is dependent on the concentration and the valency of the electrolyte.

5) Cell-surface hydrophobicity. It is quite reasonable to assume that part or all of the outer surface of some bacteria is hydrophobic. It is therefore reasonable to consider that such bacteria are rejected from the aqueous phase and attracted towards any nonaqueous phase, including solid surfaces.

1.2 Adhesion

The adhesion of bacteria to inanimate surfaces is widely recognized as having enormous ecological significance. Adhesion of microorganisms is involved in certain diseases of humans and animals, in dental plaque formation, in industrial processes, in fouling of man-made surfaces, and in syntrophic and other community interactions between microorganisms in natural habitats.

Most aquatic bacteria appear to adhere to surfaces by means of surface polymers, including lipopolysaccharides, extracellular polymers and capsules, pili, fimbriae, flagella, and more specialized structures such as appendages and prosthecae. Even though these surface components play a role in the initial, reversible adhesion, they often serve to anchor the bacterium at an interface by polymeric bridging.

The composition and quantity of bacterial cell surface polymers vary considerably and are strongly influenced by growth and environmental conditions. Although extracellular polysaccharides have been reported as being responsible for irreversible adhesion, this is not always true. For example, Brown et al.(4) demonstrated adhesion in mixed, carbon-limited populations despite no evidence of extracellular polymer production. Also, a

nitrogen-limited culture resulted in poor adhesion, although large extracellular polymer production was observed. It should always be kept in mind that polymers present between the cell and the substratum, but not observed in light or scanning electron microscopy, could be responsible for the adhesion.

It appears that, with respect to inanimate surfaces, there is a subtle balance between cell surface components that may reduce [extracellular polysaccharides, lipopolysaccharides (LPS)] or promote (fimbriae) adhesion to inanimate surfaces. Jonsson and Wadstrom (5) recently demonstrated that an encapsulated Staphylococcus aureus strain did not bind to hydrophobic Octyl-Sepharose gel, whereas a noncapsulated variant showed binding capabilities. In fact, polyanionic extracellular carbohydrate material may not be primarily concerned with the initial adhesion processes, but rather with development of subsequent bacterial film (6). The presence of LPS reduces cell surface hydrophobicity and decreases adhesion to the air-water interface of Salmonella typhimurium (7).

At short distances, many forces are important (ionic and dipolar, H-bonds, and hydrophobic interactions). Therefore, it is possible that the variabilities in bacterial surfaces result in various types of interactions that occur simultaneously with a single bacterial type. For example, hydrophobic interactions adjacent to ionic or hydrogen bonds can stabilize an otherwise energetically weak binding complex (8). Doyle et al.(8) propose that adhesion can be described in terms of positive cooperability; i.e., once the initial bacterium is bound, compounds on the substratum may change conformation, creating new receptor sites for other cells. The formation of new sites could be the result of the influence of hydrophobic interactions, but enzymatic action exposing saccharide receptors or cell-cell binding at the high bacterial concentrations used should also be considered. Unfortunately, then, theories on specificity in adhesion at inanimate surfaces and the ecological significance thereof must be discussed in view of seemingly opposing experimental data.

1.3 Adsorption of Colloidal Clays

The adsorption of colloidal clays to bacterial surfaces has been studied

by Lahav (9) and Marshall (10-12). Lahav (9) suggested that clay platelets may be oriented in a number of ways at the bacterial surface, as a consequence of the net negative charge on clay platelets and the existence of some positive charges on broken edges of platelets. Marshall (10,11) reported that species of Rhizobium with a carboxyl-type ionogenic surface sorb more Na^+-illite per cell than do species with a carboxyl-amino ionogenic surface. Using sodium hexametaphosphate (HMP) to suppress positive charges on platelet edges, Marshall (12) found that the HMP-clay did not sorb to carboxyl-type bacteria, whereas a limited amount of this clay sorbed to carboxyl-amino-type bacteria. He interpreted these results in terms of the electrostatic attraction between the platelets and the bacterial cell surfaces. Normal sodium montmorillonite particles sorb in an edge-to-face manner to carboxyl-type bacterial surfaces, with positively charged edges of the clay attracted to the negatively charged bacterial surface. Sorption in this manner is prevented by neutralization of positive edge charges of the clay by HMP. In addition to a predominantly edge-to-face association at the surface of the carboxyl-amino type-bacteria, a limited amount of face-to-face association between the negatively charged face of clay platelets and positively charged amino groups was postulated. This face-to-face association is not prevented by neutralization of the edge positive charges by HMP.

1.4 Effect of Soil Particles on Microbial Metabolism

Adsorption of organic substrates on soils depends on the nature of the particulate matter, the organization of the fabric, the clay types, and the cation status of the soils, as well as on the concentration and molecular structure of the substrate. Therefore, the availability of substrates to soil microorganisms may be enhanced or reduced by the presence of particulates.

Sugars sorb poorly to clays (13). Metabolism of glucose in soil is inversely related to the degree of bacterial sorption, which is related to the participating bacterial species (14) and the soil cation status (15). Stotzky (16,17) and Stotzky and Rem (18,19) determined that kaolinite had little effect on bacterial respiration with glucose as a substrate, whereas montmorillonite significantly stimulated respiration. Novakova (20,21) showed that the sodium and calcium forms of a montmorillonitic clay stimulated

glucose decomposition, but that these forms of kaolinite were inhibitory. Stotzky (16) also reported that when samples of montmorillonite were made homoionic to a range of cations, there was increased stimulation of bacterial respiration with saturating cation in the order Na >Ca >Mg >K >H. In a separate study, Stotzky (17) reported that bacterial respiratory activity was also related to the cation-exchange capacity and surface area of clays, but not to their particle size.

Many other excellent studies have been performed to determine the effects of clays on the decomposition of starch (22,23), aldehydes (24), pesticides (25,26), and protein (22,27,28). In most of these studies, the effect of the clay depended upon the clay type and the ionic nature of the solvent. In fact, these parameters appear to dominate the effects of clays on microbial activity.

1.5 Microbial Flocculation of Clays

In recent years several processes have been patented for the flocculation of clays, particularly clays derived from phosphate beneficiation. The use of microbial polysaccharides from such organisms as Pullularia, Xanthomonas, Arthrobacter, Cryptococcus, Hansenula, and Plectania was disclosed to flocculate finely divided inorganic solids in an aqueous medium (29). In another patent (30), the use of alkaline-treated microbial nucleoprotein is described for flocculating organic and inorganic wastes. Used in this process were nucleoproteins from the microbes Polangium, Myxococcus, Sorangium, Flavobacterium, Leuconostoc, Micrococcus, and Alcaligenes. Nucleoprotein derived from these organisms was treated with any one of a variety of alkaline compounds, including $Ca(OH)_2$, KOH, NaOH, NH_3, Na_3PO_4, and quaternary ammonium compounds, which would raise the pH to the point where the microbial material would lyse and form a sol. Flocculation of suspended waste resulted when the concentration of alkaline-treated microbial material was present in concentrations of 1 to 500 ppm (30).

Floc deterioration can result from biological factors as well as physical factors. Synthetic and natural polymers used for flocculation of colloids may be subject to degradation by microorganisms, which could result in floc

destabilization (31,32).

As can be seen from the above discussions, there are several ways in which bacteria and clay particles may interact, including attraction processes, adhesion, adsorption, the effects of colloids on microbial metabolism, and flocculation. It is entirely possible that such interactions could affect the distribution of individual particles in solution. A very simple experiment was performed in the laboratory to investigate the interaction of bacterial and clay particles and its effect on clay particle distribution. The primary focus of the experiment was to determine if bacteria are able to influence significantly the agglomeration rate of colloidal particles.

2.0 Materials and Methods

Sterile Wyoming bentonite clay (0.05 g) was added to 100 ml of sterile water. The suspension was mixed, and 1.0 ml of the suspension was added to 20 ml of nutrient broth. Three flasks containing the 20-ml nutrient broth/colloidal suspension were inoculated with a Pseudomonas sp., three of the same flasks remained uninoculated, and three flasks void of colloids were inoculated with the same bacteria. One flask void of colloids remained uninoculated, serving as a sterile control. With an Olympus Vanox microscope, these various suspensions were monitored for over 200 hours. Individual colloids, and agglomerates (clusters) containing 2 to 5, 6 to 10, 10 to 25, and >25 particles, were counted. Counts were made with a Petroff-Hausser bacteria counting chamber having a volume of 1/400 mm^2 by 1/50 mm deep, or 5×10^{-5} mm^3, or 5×10^{-8} ml. This experiment was repeated three times; the results are presented in Figures 1-8 as counts per 5×10^{-8} ml.

3.0 Results and Discussion

These results clearly demonstrate that the presence of bacteria significantly influenced the rate of colloidal agglomeration. In the presence

of bacteria, the number of individual clay particles decreased rapidly with time (Figure 1). However, in the absence of bacteria (sterile) (Figure 6), the number of individual clay particles increased with time, presumably because of the disruption of preformed agglomerates present at time 0. Both the 2- to 5- and 6- to 10-particle clusters tended to increase with time in the presence of bacteria, while the 10- to 25- and >25-particle clusters followed a more complicated function, first increasing and then decreasing with time.

Sterile clay suspensions followed a more predictable pattern. Generally, the preformed cluster groups (both 2 to 5 and 6 to 10) tended to disassociate with time, as indicated by the increase in single particles (Figure 6). The inoculated, noncolloid flasks displayed a fairly typical growth curve, and therefore the results are not presented. The sterile control remained sterile throughout each of the experiments.

From the results, one can clearly see that the presence of bacteria profoundly affected the distribution of particles in suspension. These results are extremely important to the NNWSI Project. As previously mentioned colloidal dispersion has been implicated in the transport of radioactive wastes in soil systems. Microorganisms (both indigenous and exogenous) will exist in the vicinity of the candidate site of the high-level nuclear waste repository. The results of this study suggest that an interaction will occur between these bacteria and colloidal particles. As a consequence, colloidal dispersion of actinide elements could be influenced by the agglomeration of colloidal particles.

4.0 Future Accomplishments

This laboratory experiment demonstrated that there exists a strong potential for microorganisms to affect colloidal particle distribution. However, it was not quantitative in design and therefore can only suggest the possibility of strong interactions between microorganisms and colloids. It is necessary to describe the mechanism(s) by which microorganisms influence the

agglomeration of colloidal particles. Although the literature contains numerous studies that discuss the interactions of bacteria and colloids, none of the studies address colloidal agglomeration, as affected by bacteria. Hence, there exists little information regarding (1) agglomeration rate, (2) agglomerate stability, and (3) particle distribution within the agglomerate. The approach of future research is to conduct a series of experiments addressing each of these issues.

4.1 Agglomeration Rate

A key to understanding the bacterial influence of colloidal agglomeration is the determination of agglomerate formation kinetics. Although the preliminary experiments demonstrated the involvement of bacteria in the agglomeration process, they provided no kinetic information. Additional experiments need to be performed to establish the rate of agglomerate formation. This information would be very useful in understanding the types of attractions that are occurring between the clay and bacterial particles. For example, agglomerate formation occurring parallel to bacterial growth would suggest that agglomeration is simply a function of the number of bacterial particles. If, however, agglomeration is delayed until stationary growth phase, then one would suspect that agglomeration is "threshold" in nature and depended upon some other parameter, such as pH or the concentration of extracellular metabolic products.

In a related issue, it would also be useful to determine if a threshold concentration of clay particles is needed to initiate aggregate formation. Only one clay concentration was used in the preliminary experiments; however, in the proposed experiments, the concentration of clay particles will be varied.

4.2 Agglomerate Stability

Another important aspect of the proposed research is to study the stability of the bacterial/clay agglomerate. From the preliminary studies one can observe that the number of particles within an agglomerate cluster changes with time. It appears that the large clusters are forming early, followed by a breakdown into the smaller cluster units (2 to 5 and 6 to 10); however,

more detailed studies need to be performed to determine the interaction among these cluster units.

In addition, the long-term stability of the clusters should be studied. Such studies should address the eventual fate of the clusters: is there one particular cluster unit that will dominate? Will there be an equal distribution of particles among all the cluster units or will all the clusters eventually break down to individual particles?

4.3 Particle Distribution Within the Agglomerate

The relative distribution of bacteria and clay particles within a single agglomerate should be determined. It is not known whether the ratio of bacteria to clay is uniform among all the cluster units, or if this ratio changes with the size of the cluster. It may be that single bacterium initiates cluster formation, or several bacteria in close proximity to one another may be responsible for aggregate formation.

4.4 Experimental Procedure

As in the preliminary studies, light microscopy will be used to analyze the colloid/bacteria interactions. An Olympus Vanox Research microscope equipped with two 35-mm cameras and one 4x5 polaroid camera will be used for the analyses. For most of the observations, either phase contrast or epi-fluorescence microscopy will be used. In addition, the microscope will be equipped with a Sanyo VDC 3800 video camera having a digital analog converter. Image analysis will be done by a PC Vision Frame Graber coupled with an IBM-PC using an appropriate software program. Particle and cluster analysis can therefore be performed much more quickly than was done in the preliminary studies, and data storage will be automated.

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Figure 1. Number of individual clay particles per 5.0×10^{-8} ml in the presence of a Pseudomonas sp.

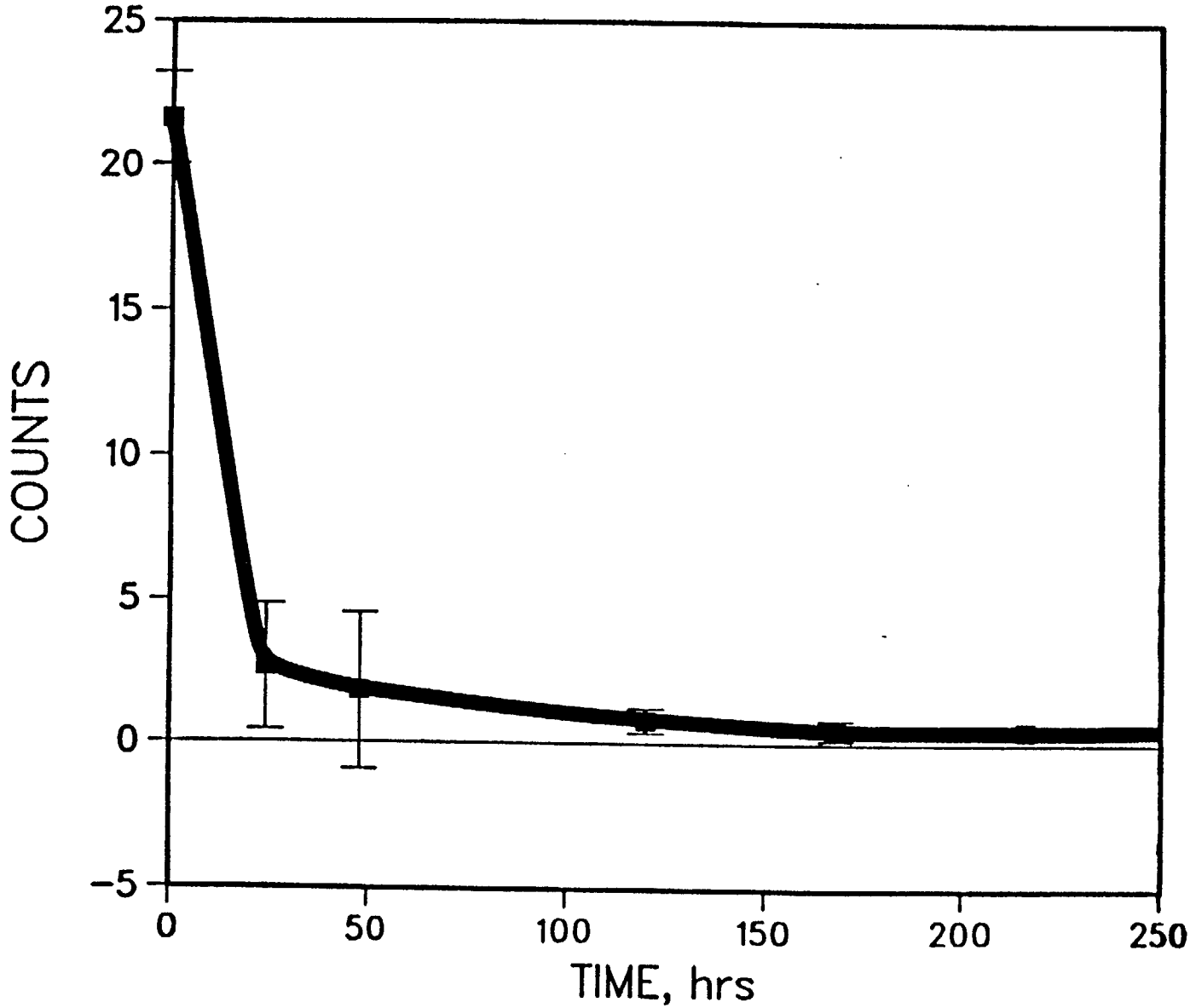

Figure 2. Number of clusters per 5.0×10^{-8} ml, containing 2 to 5 clay particles, in the presence of a Pseudomonas sp.

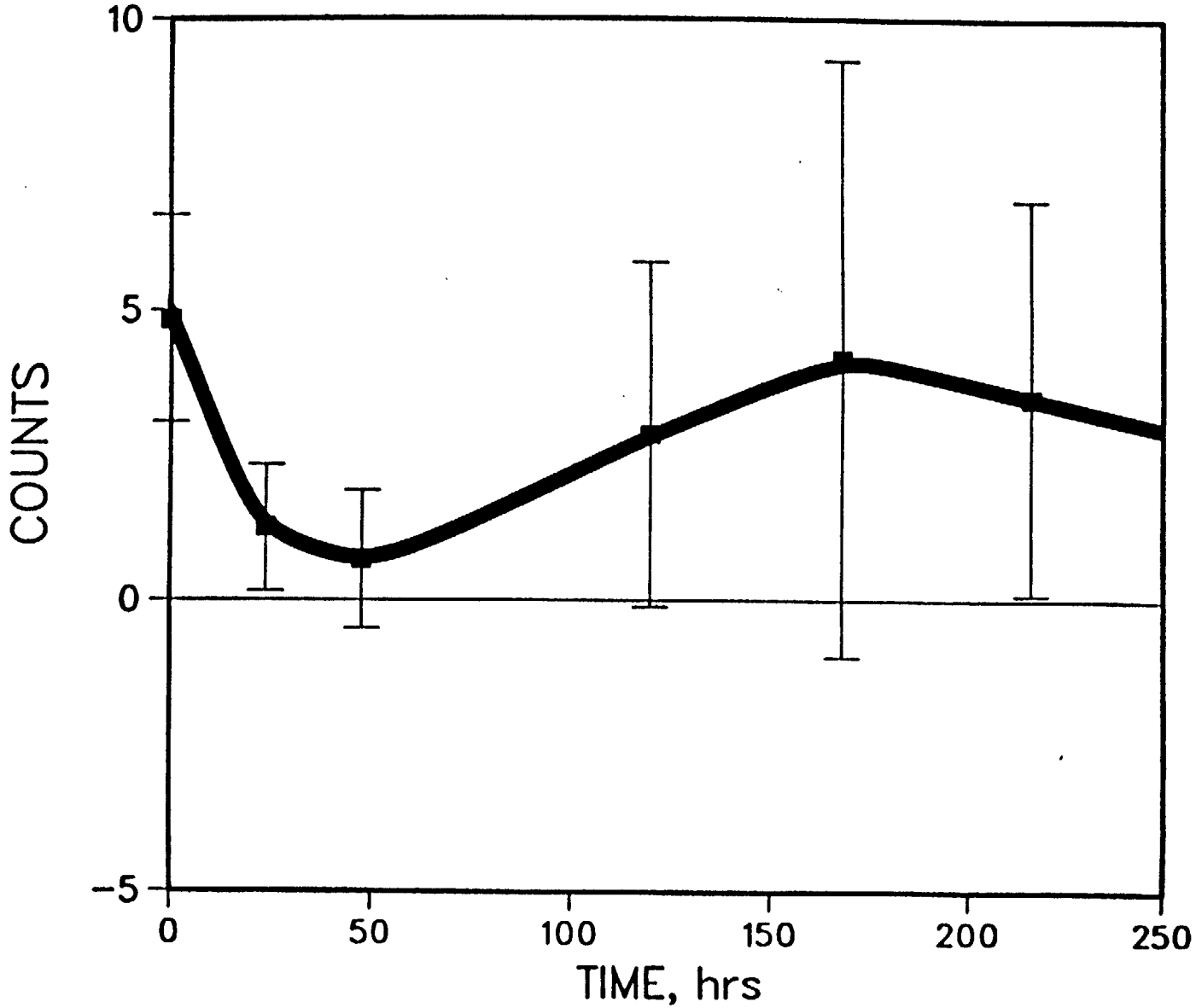

Figure 3. Number of clusters per 5.0×10^{-8} ml, containing 6 to 10 clay particles, in the presence of a Pseudomonas sp.

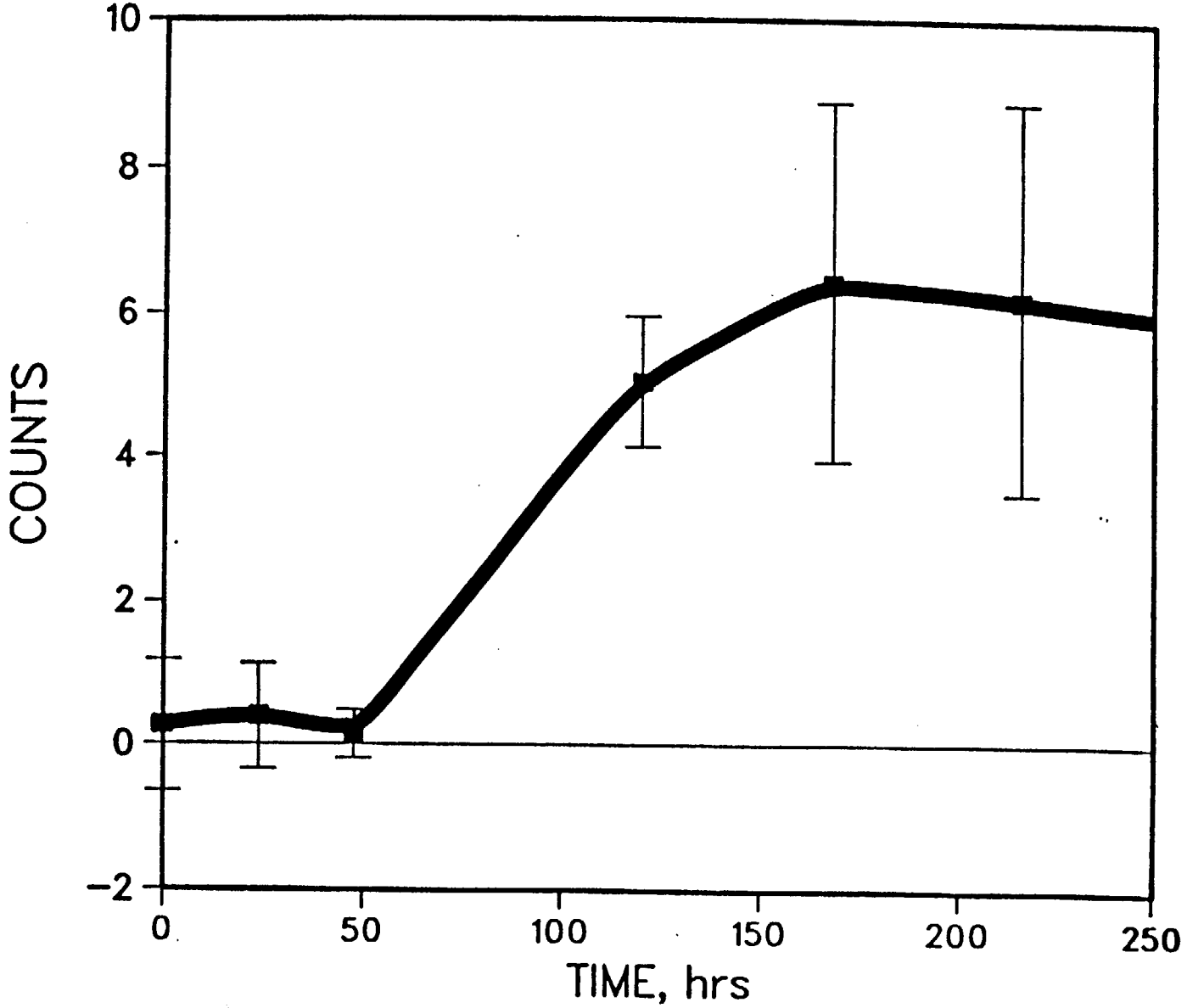

Figure 4. Number of clusters per 5.0×10^{-8} ml, containing 10 to 25 clay particles, in the presence of a Pseudomanas sp.

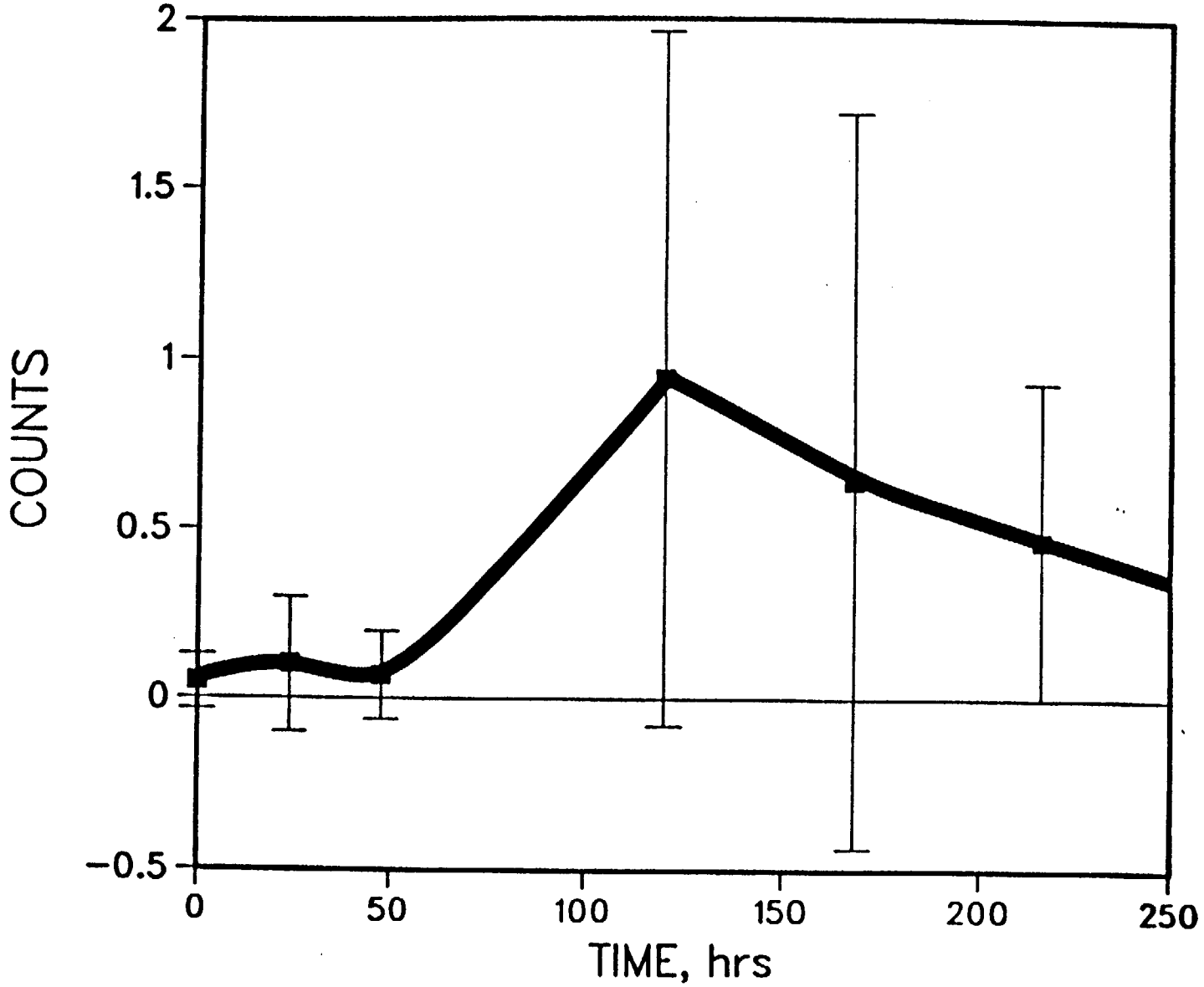

Figure 5. Number of clusters per 5.0×10^{-8} ml, containing greater than 25 particles, in the presence of a Pseudomonas sp.

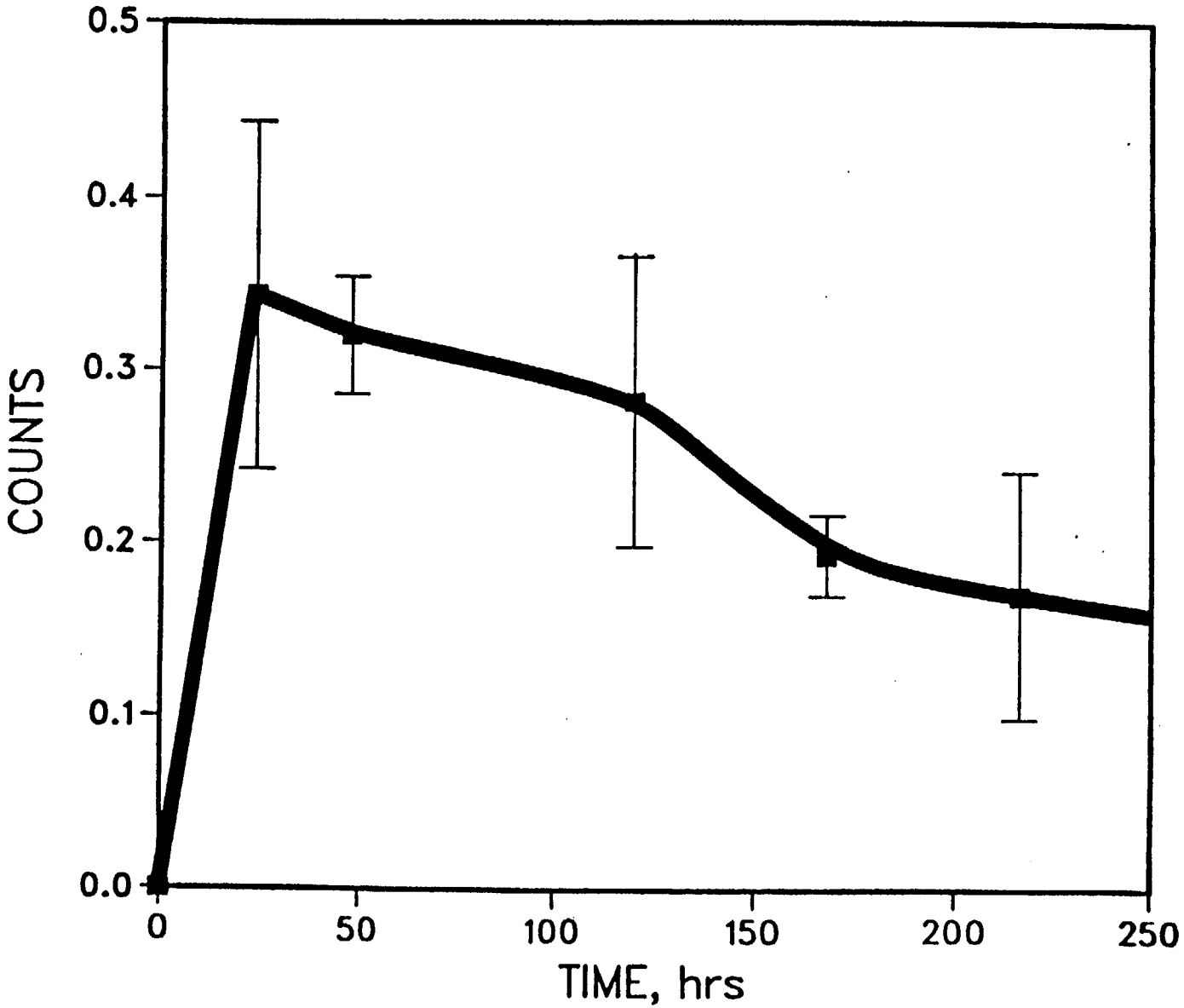

Figure 6. Number of clay particles per 5.0×10^{-8} ml, in sterile nutrient broth.

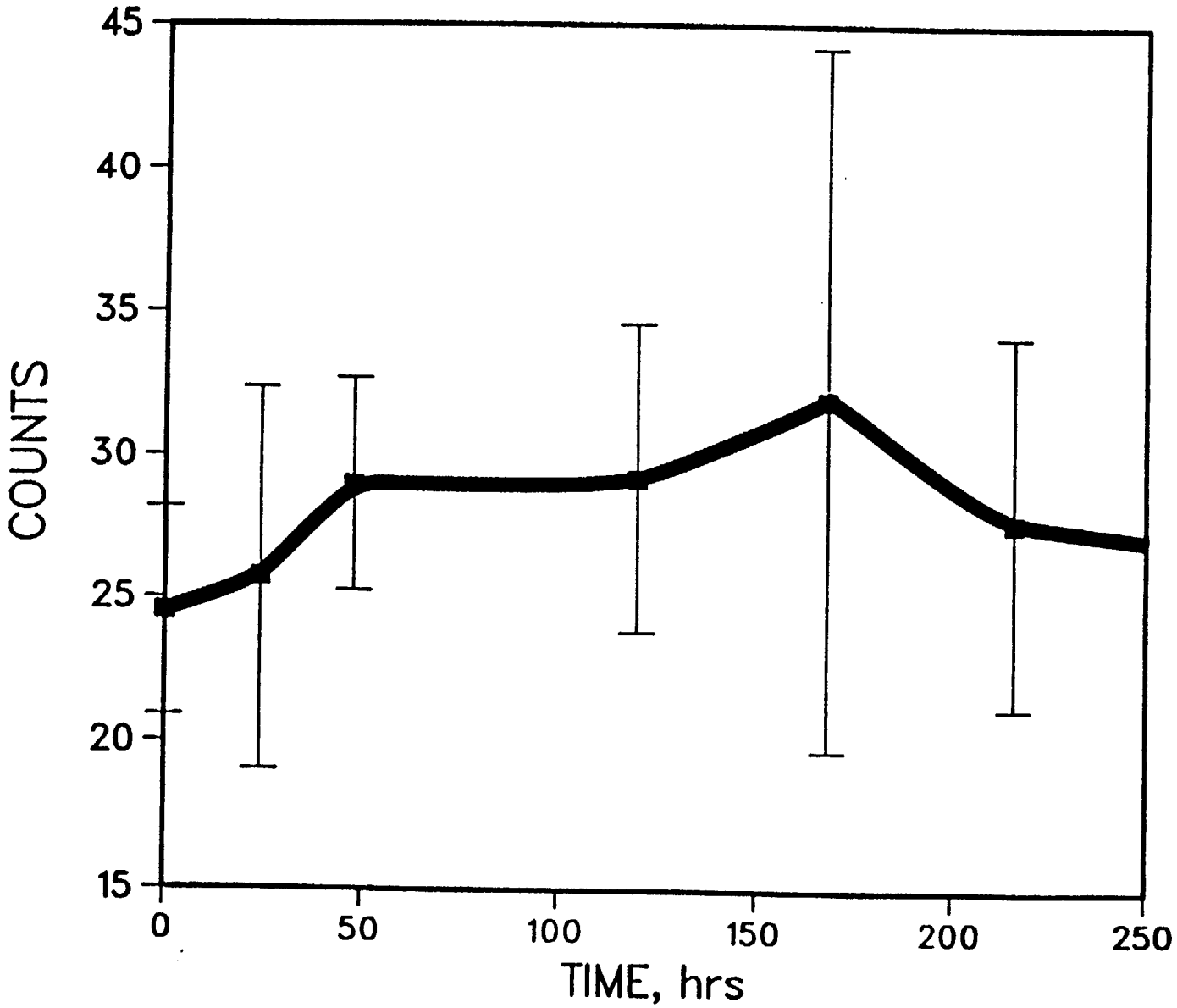

Figure 7. Number of clusters per 5.0×10^{-8} ml, containing 2 to 5 clay particles, in sterile nutrient broth.

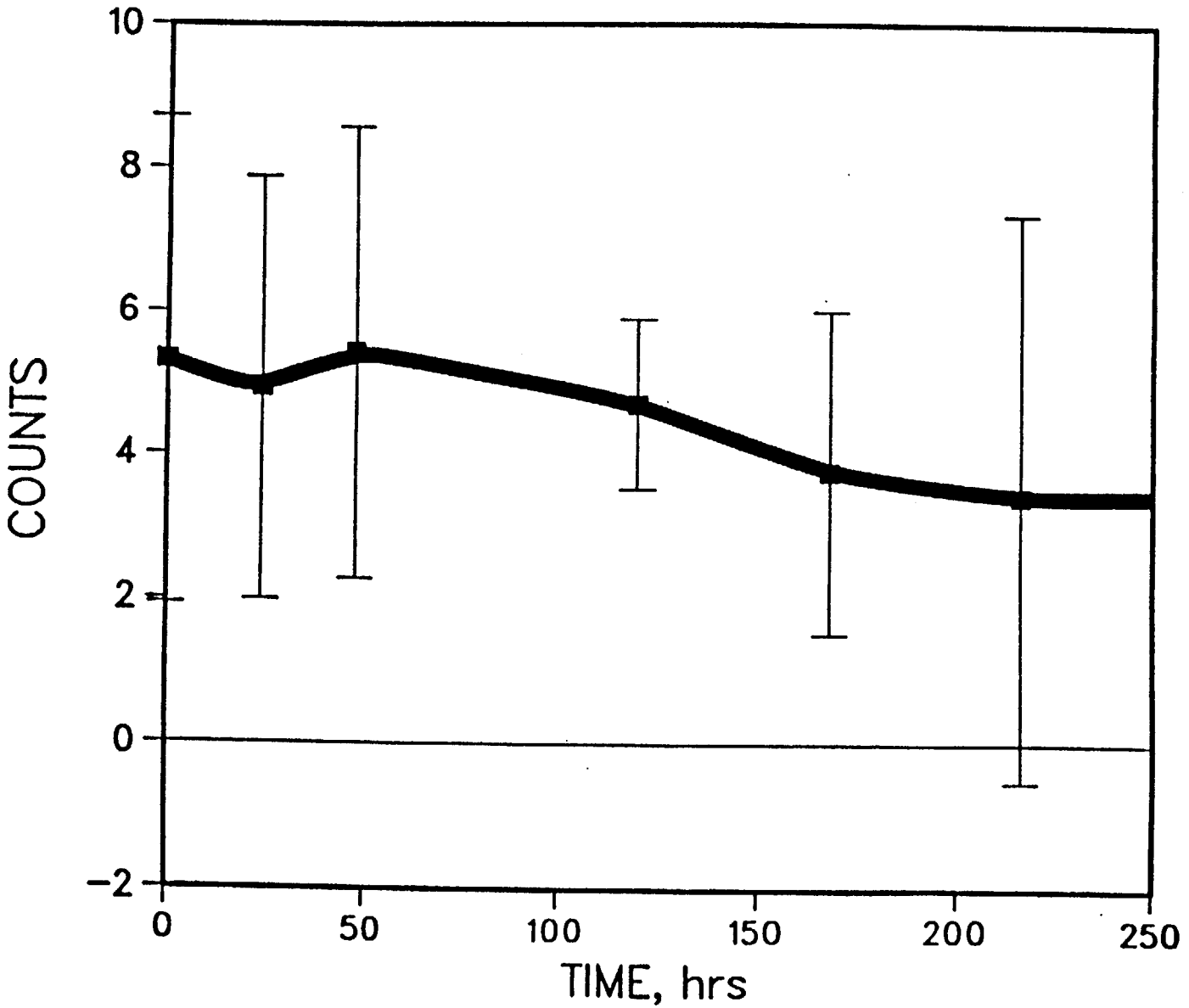

Figure 8. Number of clusters per 5.0×10^{-8} ml, containing 6 to 10 clay particles, in sterile nutrient broth.